Functional Implications of the Regulation of PASKIN mRNA

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1 ZUSAMMENFASSUNG

Änderungen in der Umwelt zu detektieren und darauf zu reagieren ist eine Schlüsseleigenschaft des Lebens. In allen Bereichen des Lebens führen PER-ARNT-SIM (PAS) Domänen enthaltende Proteine oft beide dieser Funktionen aus. PAS-Domänen sind befähigt einen Stimulus direkt oder über die Bindung eines Kofaktors zu detektieren und dieses Signal an Effektor-Domänen weiterzuleiten. PAS-Domänen können auch als Protein-Protein Interaktions-Domänen für die Heterodimerisierung von Transkriptionsfaktoren fungieren. Oft sind diese Transkriptionsfaktoren auch in einen Sensorprozess involviert. Animalia besitzen ein einziges Gen, welches für eine einzigartige Kombination einer Serin/Threonin-Kinase-Domäne mit einer PAS-Domäne kodiert, obwohl in Pflanzen und Bakterien die Kombination einer Kinase mit einer PAS-Domäne häufig vorkommt. Die Säugetier PAS Serin/Threonin-Kinase wurde durch die Ähnlichkeit ihrer Sequenz zur bakteriellen Histidinkinase FixL identifiziert und wurde PASKIN genannt. Die beiden Orthologe von PASKIN in der Hefe sind in den Kohlenhydratmetabolismus und wahrscheinlich in die Proteintranslation involviert. Ausserdem spielen sie eine Rolle für die Integrität der Zellwand. Anscheinend ist die Rolle von PASKIN im Energiemetabolismus von der Hefe bis zum Menschen konserviert. Es wurde gezeigt, dass PASKIN als Antwort auf eine Änderungen der Glukosekonzentration die Sekretion von Insulin und Glukagon in β- und α-Zellen des Pankreas reguliert. Der Effekt eines Verlusts von Paskin auf den gesamten Organismus wurde mit Hilfe von *Paskin* Knockoutmäusen untersucht. Obwohl unter normalen Tierhaltungsbedingungen keine Unterschiede im Phänotyp verglichen mit Wildtyptieren beobachtet wurden, gab es Berichte, dass unter fetthaltiger Ernährung *Paskin* Knockoutmäuse vor den Schäden des metabolischen Syndroms teilweise geschützt sind. Mäuse ohne Paskin schneiden in Glukose- und Insulintoleranztests unter fetthaltiger Ernährung besser ab als Wildtypmäuse, und sie legen weniger an Körpergewicht zu als ihre Wildtypgeschwister. Wie auch immer, wir konnten diese Effekte nur teilweise reproduzieren, obwohl wir das gleiche Tiermodell benutzten.

In Mäusen ist die Konzentration der PASKIN mRNA abgesehen vom Hoden, am höchsten in Geweben, die mit dem Immunsystem assoziiert sind. Ziele dieser Dissertation waren den Grund für diese Beobachtung zu bestimmen und zu untersuchen wie die Expression von PASKIN reguliert wird. Wir zeigen hier, dass das Gewebeexpressionsmuster von PASKIN im Menschen konserviert ist und dass dieses auf hohen mRNA Konzentrationen in der Lymphozytenlinie beruht. In einer Serie von Experimenten mit Leukozytenzelllinien und

primären mononukleären Zellen aus dem peripheren Blut (PBMCs), die wir mit Entzündungsmediatoren stimuliert haben, haben wir eine möglich Regulation von PASKIN in der Immunantwort untersucht. Wir haben entdeckt, dass die Konzentration für PASKIN mRNA während der Aktivierung von Zelllinien und primären T- und B-Zellen stabil bleibt, was eine mögliche Funktion für PASKIN eher während als nach der Aktivierung vermuten lässt. Desweiteren zeigen wir, dass die Leukozytenspezifität der Expression von PASKIN abhängig ist von einer Promotorregion, die mit der regulatorischen Untereinheit 7 der Proteinphosphatase 1 (*PPP1R7*) geteilt wird. Zudem waren wir auch an der evolutionären Konservierung dieses bidirektionalen Promotors interessiert. Obwohl wir entdeckten, dass PASKIN in allen Animalia konserviert ist, ausser der bemerkenswerten Ausnahme von Nematoden, ist der konservierte bidirektionale Promotor nur im Fleischflossser *Latimeria chalumnae* und in Tetrapoden zu finden. Da andere Fische keine Konservierung der genomischen Lokalisation von *PASKIN* und *PPP1R7* aufwiesen, könnte dies vermuten lassen, dass diese Konstellation während der Eroberung des Festlandes für Wirbeltiere von Vorteil gewesen sein könnte. Eine mögliche Koregulation von PASKIN und PPP1R7 könnte ein Hinweis auf eine funktionelle Verbindung zwischen diesen beiden Genen sein. Das Hefeortholog von PPP1R7 ist im mitotischen Kontrollpunkt involviert und kürzlich wurde auch Paskin in *Drosophila* mit dem Spindelaufbaukontrollpunkt und mit, durch Schäden der DNA induzierte, Apoptose in Verbindung gebracht. Deshalb haben wir eine mögliche Verbindung zum Spindelaufbaukontrollpunktprotein Mad2 in Säugetierzellen untersucht. Wir beobachteten, dass in Säugetierzellen die Anfälligkeit für Schäden der DNA und die Antwort auf das Zellgift Nocodazol unabhängig von Paskin sind. Mittels Immunfluoreszenz konnten wir sehen, dass PASKIN mit dem Aktinzytoskelett kolokalisiert, was funktionell bedingt könnte. Zusammen mit unseren Entdeckungen über eine mögliche Koregulation von PASKIN und PPP1R7 weisst dies auf bisher unbekannte Funktionen von PASKIN hin.

2 SUMMARY

Sensing and reacting to environmental changes is a key feature of life. PER-ARNT-SIM (PAS) domain-containing proteins often execute both these tasks at the molecular level throughout all kingdoms of life. PAS domains are able to sense a stimulus directly or through binding of a cofactor and transduce these signals to effector domains. PAS domains can also work as a protein-protein interaction domain for the heterodimerisation of transcription factors. Often these transcription factors are still involved in sensing processes. Animalia possess one single gene encoding a unique serine/threonine kinase combined with a PAS domain, although the combination of a PAS domain with a kinase domain is common in bacteria and plants. The mammalian PAS serine/threonine kinase has been identified by sequence similarity to the bacterial histidine kinase FixL and has been named PASKIN. In yeast, two orthologs of PASKIN are known to be involved in carbohydrate metabolism, cell wall integrity and possibly translation. From yeast to human, the involvement of PASKIN in energy metabolism is apparently conserved. It has been shown that in response to changes in glucose concentration PASKIN regulates insulin and glucagon secretion in pancreatic β- and α-cells, respectively. The effect of a loss of Paskin on the whole organism has been investigated using *Paskin* knockout mice. Although under normal husbandry conditions no differences have been observed compared to wildtype animals, *Paskin* knockout mice were reported to be partially protected from the detrimental effects of the metabolic syndrome under high fat feeding conditions. Mice lacking Paskin show a subtle increase in their performance in glucose and insulin tolerance tests under high fat feeding conditions compared to wildtype mice and they gain less bodyweight than their wildtype littermates. However, despite using the same animal model, we could only partially reproduce these effects observed in other labs.

In mice, Paskin mRNA levels are, apart from testis, high in tissues associated with the immune system. Goals of this thesis were to determine the reason for this observation and how PASKIN expression is regulated. Herein, we demonstrate that the mouse Paskin mRNA expression pattern is conserved in human and that it is due to high PASKIN mRNA levels in the lymphocyte lineage. This raised the question if PASKIN might be regulated during the activation of T- and B-cells. We investigated a putative regulation of PASKIN in the immune response in a series of experiments in leukocyte cell lines and primary peripheral mononuclear cells (PBMCs) stimulated with pro-inflammatory stimuli. We found that PASKIN mRNA and protein levels remain stable during the activation of cell lines and

primary T- and B-cells, suggesting the putative role for PASKIN during the activation and not after. We further show that the leukocyte-specific expression of PASKIN depends on a promoter region which is shared with the protein phosphatase 1 regulatory subunit 7 (*PPP1R7*). We were interested in the evolutionary conservation of this bidirectional promoter. Although PASKIN was found to be conserved throughout the animal kingdom, with the peculiar exception of nematodes, we found the bidirectional promoter of *PASKIN* and *PPP1R7* is conserved in tetrapods and the lobe-finned fish *Latimeria chalumnae*. Since other fish do not show this genomic localisation of *PASKIN* and *PPP1R7* this might suggest this constellation was beneficial during terrestrialization of vertebrates. A putative co-regulation of PASKIN and PPP1R7 might indicate a functional connection of these two genes. Since the yeast ortholog of PPP1R7 is involved in the mitotic checkpoint and Paskin has recently been associated with the spindle assembly checkpoint and DNA damage-induced apoptosis in *Drosophila*, we studied a putative connection between PASKIN and the spindle assembly checkpoint protein MAD2 in mammalian cells. We found that susceptibility to DNA damage and the response to nocodazole is independent of PASKIN in mammalian cells. By immunofluorescence we found PASKIN partially colocalised with the cytoskeleton, which might have functional consequences for PASKIN. Together with our findings of a putative co-regulation of PASKIN and PPP1R7 this implicates so far unknown functions for PASKIN.

3 INTRODUCTION

3.1 PAS domain proteins

Adaptation to alterations in the environment requires the ability of sensing these changes. Various specialized molecules sense environmental changes and activate signalling cascades resulting in an adequate response to the perceived signal. One of the many evolved designs for such sensor molecules are PAS domain proteins. The Per-Arnt-Sim domains are named after the *Drosophila* circadian rhythm protein Period (Per), the aryl hydrocarbon receptor nuclear translocator (Arnt) and the *Drosophila* protein single-minded (Sim) (Hoffman et al. 1991). PAS domains share similarities mainly on the structural level, whereas they show a rather low sequence identity of below 20% on average (Finn et al. 2006). PAS domain proteins consist of a single or of multiple PAS domains that are labelled alphabetically starting with A from the N to the C terminus. A typical PAS domain displays a single antiparallel 5-stranded β-sheet and five alpha helices often forming a pocket like structure that is used for cofactor binding (Gong et al. 1998; Taylor and Zhulin 1999). However, for many PAS domain proteins a cofactor has not been identified and it is questionable if such a cofactor exists for every member of the PAS protein family (Möglich et al. 2009).

The PAS domain proteins acting as sensors are able to detect stimuli of a wide variation, ranging from oxygen over light to changes in membrane potential. Most of the identified PAS domain proteins act in signal transduction. Therefore PAS domains are usually combined with effector domains enabling the transduction of the received signal. Almost half of all identified PAS domains are combined with a histidine kinase domain (Henry and Crosson 2011). This shows the unequal distribution of PAS- domain proteins over the different kingdoms of life considering that higher animals do not have histidine kinases (Plowman et al. 1999; Koretke et al. 2000). However, PAS domain proteins are as diverse in function as DNA binding proteins and potassium ion channels.

In addition to play the part of a sensor, PAS domains can function as a protein-protein interaction domain. One of the best studied proteins making use of the PAS-PAS interaction ability are the-subunits of the transcription factor hypoxia inducible factor 1 (HIF-1) (Wang et al. 1995). HIF-1 is a heterodimeric transcription factor regulating gene expression in response to hypoxia. It has been shown that the heterodimerisation between the subunits HIF-1α and ARNT or HIF-1β via their PAS domains promotes stability and DNA binding (Kallio et al. 1997). The heterodimerisation is not a requirement for nuclear entrance of HIF-1 (Chilov et

al. 1999). HIF-1 acts downstream of oxygen sensing prolyl hydroxylases (PHDs). Under normal oxygen concentrations HIF-1α subunit is constantly marked by ubiquitination and degraded in the 26S proteasome (Huang et al. 1998). The signal inducing this ubiquitination is the hydroxylation of HIF-1α by the PHDs (Bruick and McKnight 2001; Epstein et al. 2001). The hydroxylation of specific proline residues in the C-terminal so called oxygen dependent degradation domain (ODD domain) are recognized by the von Hippel-Lindau tumor suppressor protein (VHL) as part of an E3 ubiquitin ligase complex (Lisztwan et al. 1999; Maxwell et al. 1999; Ivan et al. 2001; Jaakkola et al. 2001). The reduced availability of the hydroxylase substrate oxygen results in a decreased hydroxylation of HIF-1α, thus evading degradation in the proteasome. Subsequently, the HIF-1α-ARNT/HIF-1β heterodimer is formed. This leads to the activation of transcription of genes relevant under hypoxic conditions.

Another PAS domain protein involved in oxygen sensing is FixL a histidine kinase regulating the expression of genes involved in nitrogen fixation in *Bradyrhizobium japonicum*. Low oxygen concentrations are critical for the function of nitrogenase. Unlike HIF-1α, FixL is able to bind oxygen directly. In FixL this is achieved by a heme incorporated in the pocket structure typical for PAS-domains. Binding of oxygen to the heme induces a conformational change of the PAS domain. The main change occurs in a so called regulatory loop linking the Gβ strand and the Fα helix (Gilles-Gonzalez and Gonzalez 2005). The binding of oxygen leads to the inactivation of the histidine kinase activity of FixL and ultimately to a reduced phosphorylation of the transcription factor FixJ (Gilles-Gonzalez et al. 1991). FixJ is regulating nitrogen fixation genes (David et al. 1988).

3.2 The PAS domain containing serine/threonine kinase PASKIN

A BLAST search with the FixL sequence of *Bradyrhizobium japonicum* identified a single eukaryotic homolog to FixL in human (Hofer et al. 2001). This PAS domain containing serine/threonine kinase was named PASKIN. Further sequence homology searches identified an ortholog in *Drosophila* and two orthologs in yeast. The constellation of the two PAS domains and the serine/threonine kinase domain is conserved from yeast to human (Rutter et al. 2001). Unfortunately, two different names were proposed for the mammalian PAS domain containing serine/threonine kinase. Out these two, *PASKIN* is preferable because it is unique among all the designations of mammalian genes. The designation PASK proposed by Rutter

and co-workers is also used for another serine/threonine kinase proline-alanine-rich Ste20-related kinase (STK39 or PASK) (Ushiro et al. 1998). Considering the same molecular function and the coincidental involvement of STK39 in pancreas development, it is not surprising that the unfortunate choice of PASK instead of PASKIN have led to confusions (Miao et al. 2000). Therefore, the distinctive name PASKIN will be used throughout this work.

3.3 The two yeast orthologs of PASKIN

In yeast, the two orthologs of PASKIN are named Psk1 and Psk2. Under stress conditions double knockout mutants of these two genes result in a strongly reduced ability to grow on medium providing only galactose as a carbon source (Rutter et al. 2002). Interestingly, UDP-glucose pyrophosphorylase 1 (Ugp1) was identified to be phosphorylated by Psk2. Ugp1 is catalysing the reaction of UTP and glucose-1-phosphate to UDP-glucose and pyrophosphate an important step in the storage carbohydrate synthesis (Daran et al. 1995). Psk2 mutant strains are hypersensitive to Ugp1 overexpression but not to the overexpression of a non functional form of Ugp1. Increased levels of a mutant Ugp1, mimicking a phosphorylation of serine 11, result in a growth defect independently of the *Psk1* and *Psk2* allelic background. Together, these observations suggest a role for Psk2 in regulation of Ugp1 activity. In accordance with these findings, *Psk2* and *Psk1* mutants show excess glycogen accumulation (Rutter et al. 2002). The same group showed that, in addition to a lack of Ugp1 phosphorylation, unpaired phosphorylation of glycogen synthase (Gsy2p) in *Psk2* mutants seems to add to this accumulation of storage carbohydrates.

Intriguingly, Psk2 dependent phosphorylation of Ugp1 is increased when the cell integrity is compromised by sodium dodecyl sulphate (SDS), calcofluor white or chlorpromazine (Grose et al. 2007). In yeast, a family of cell integrity pathway genes called *Wsc* (for cell wall stress response component) are required for cell wall integrity (Verna et al. 1997). Although, overexpression of Wsc1 increases phosphorylation of Ugp1, *Wsc1, Wsc2* and *Wsc3* are not essential for cell integrity stress induced phosphorylation of Ugp1 (Grose et al. 2007). The cell integrity stress induced phosphorylation of Ugp1 has been found to be mainly Psk2 dependent. On the other hand non-fermentative carbon sources like ethanol, glycerol or rafinose stimulate Ugp1 phosphorylation by Psk1 in a Snf1 dependent way. The AMPK ortholog Snf1 is a key regulator of the switch in gene expression upon change of carbon

source (Gancedo 1998; Young et al. 2003). Both Psk1 and Psk2 are activated post-translationally under the described stress conditions. The activation is dependent on the PAS domain containing N-terminus (Grose et al. 2007). The phosphorylation of Ugp1 at Ser11 induced by both pathways does not affect enzymatic activity of Ugp1. Instead, Ugp1 undergoes a conformational change. This leads to an increased production of structural carbohydrates like glucan instead of storage carbohydrates. It was suggested that the conformational change leads to a reallocation of Ugp1 to the cell membrane where glucan synthesis primarily takes place (Smith and Rutter 2007).

In addition, it has been shown that Psk2 or Psk1 dependent phosphorylation of Ugp1 induces the formation of a complex with suppressor of Sit4 deletion (Ssd1) and Rho1 multicopy suppressor 2 (Rom2). Rom2 is known as a guanine nucleotide exchange factor (GEF) specific for RAS homolog 1 (Rho1). The function of Ssd1 remains elusive. The Ugp1-Ssd1-Rom2 complex is able to activate Rho1 (Cardon et al. 2012). The small GTPase Rho1 is implicated in activating the MAPK pathway (Nonaka et al. 1995). Additionally, Rho1 is involved in cell wall synthesis and cytoskeleton organisation (Qadota et al. 1996; Imamura et al. 1997).

Others have suggested that the ability of yeast Psk2 to regulate carbohydrate metabolism is important to maintain ultradian clock-coupled respiratory oscillation (UCRO) in yeast (Ouyang et al. 2011).

In addition to Ugp1, Cap-associated factor 20 (Caf20), eukaryotic translation initiation factor 4E (eIF4E) and suppressor of rho 3 deletion 9 (Sro9) have been identified to be phosphorylated in a Psk2-dependent manner (Rutter et al. 2002). All three of which are involved in regulation of protein synthesis. However, in yeast little to none research was done in this direction. Intriguingly, the *Candida albicans* ortholog of Psk2 has been identified as a negative regulator of silent information regulator 2 (Sir2) (Raisner and Madhani 2008). Sir2 is also called sirtuin. From yeast to human sirtuins are involved in a series of cellular processes including metabolic regulation, longevity and apoptosis.

Although, some aspects of the downstream function of Psk1 and Psk2 are understood the exact mechanism of the activation of the kinase domains is still unknown. In fact, a regulatory metabolite acting as a ligand for the PAS domain has been suggested (Grose et al. 2009). However, until now no such ligand has been identified. Some of the processes for which a relation to the PASKIN orthologs has been indicated might be conserved in mammals. However, mammalian cells do not have a cell wall what in combination with their higher

complexity result in different requirements to the regulation of energy metabolism and carbohydrate utilisation. Therefore, although the yeast PAS kinase orthologs may show some similarities to the mammalian PASKIN, it is questionable how much of PASKIN function is conserved.

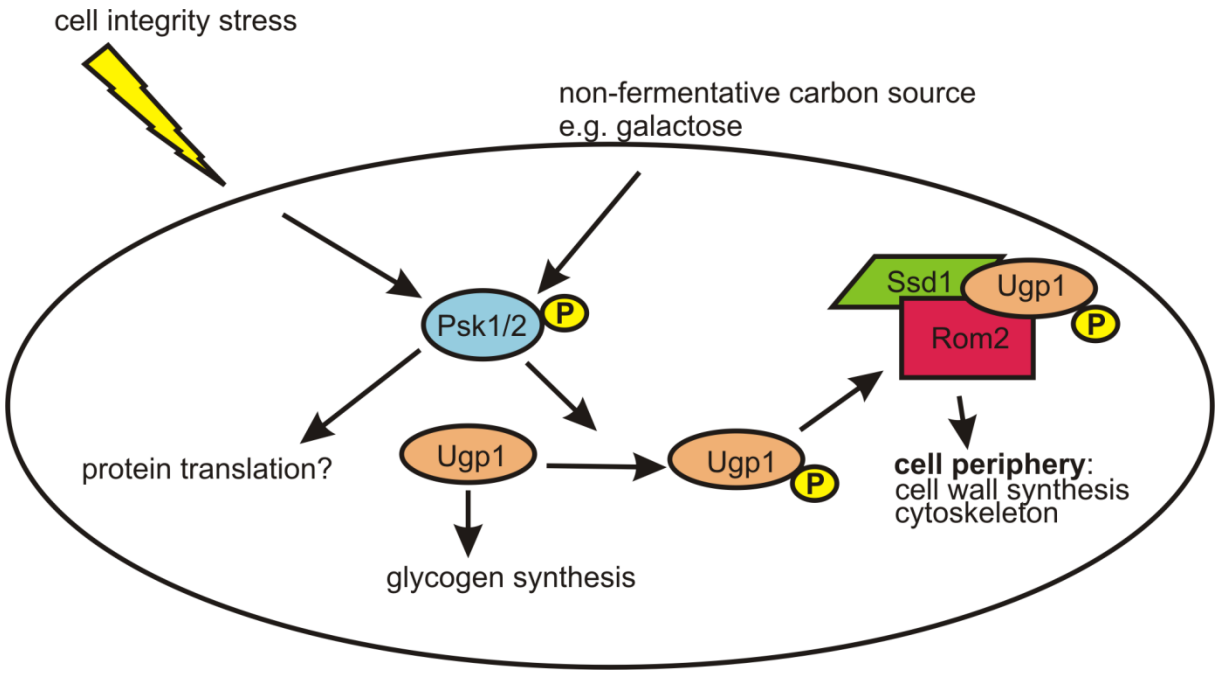

Fig. 1. Summary of the current understanding of the biological functions of Psk1 and 2, in yeast. Cell integrity stress or growth on a non fermentative carbon source like galactose leads to the activation of Psk1 and 2. Both phosphorylate Ugp1 which leads to a conformational change of Ugp1 and its relocalisation to the cell periphery. The relocation leads to a switch from glycogen synthesis to glucan synthesis. Glucan is a component of the cell wall. Recent findings suggest Ugp1 to form a complex with Ssd1 and Rom2 upon phosphorylation and therefore to be involved in cytoskeleton organisation.

3.4 The interplay between structure and activity of PASKIN

3.4.1 The PAS-domain

The crystal structure of the PAS A domain of human PASKIN has been determined by nuclear magnetic resonance (NMR) experiments (Amezcua et al. 2002). The crystal structure of the N-terminal PAS domain of the mammalian PASKIN shows the typical PAS domain fold of five antiparallel β-sheets flanked by α-helices. Additionally, the PAS domain of PASKIN has a flexible loop between the Fα and the Gβ strand. This resembles the region of

the FixL regulatory loop. The high flexibility of this loop region in the PASKIN PAS domain suggests the binding of a putative ligand. Indeed, Amezcua and colleagues found, by screening a library of 750 organic compounds, that preferably hydrophobic compounds consisting of two aromatic rings separated by a short linker bind to the PAS domain. The different compounds have various polar substitutes distributed around their aromatic rings. Interestingly, the PAS domain of PASKIN has been found to inhibit the activity of the PASKIN kinase domain in cis and in trans *in vitro* (Rutter et al. 2001). Based on these observations, a model was proposed in which a ligand binds to the PAS A domain of PASKIN and by that dis-inhibits the PASKIN kinase domain. Unfortunately, no endogenous ligand binding the PAS domain could be identified so far.

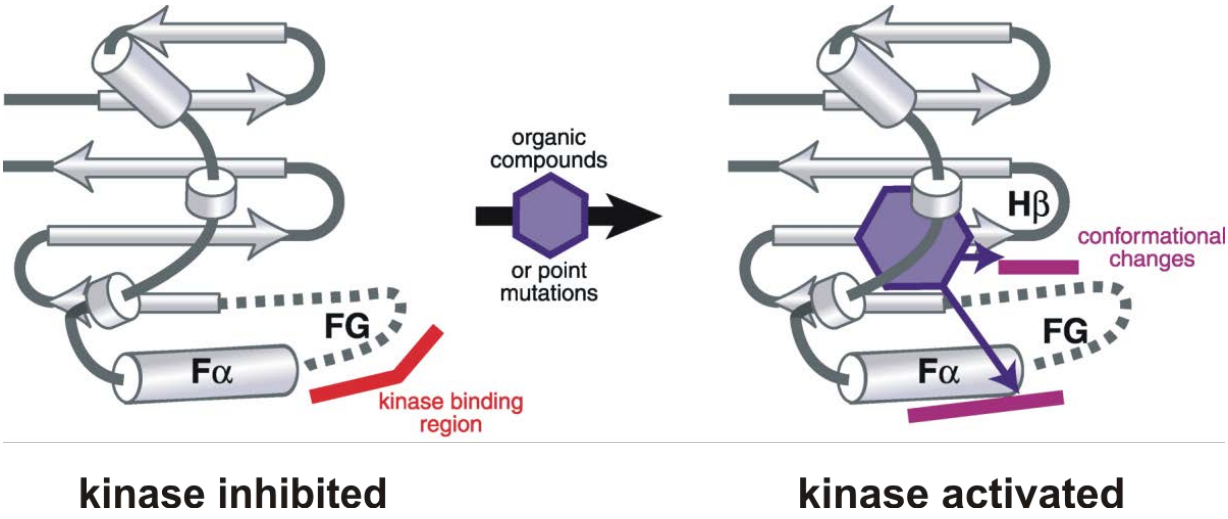

Fig. 2. Model for PAS domain control of kinase activity of PASKIN. Upon binding of a yet unknown ligand the PAS domain releases the kinase domain. This leads to increased kinase activity and therefore to increased auto- and target-phosphorylation (Amezcua et al. 2002).

3.4.2 The kinase domain

The PASKIN kinase domain shows structural relation to AMP-activated protein kinase α and β (AMPKα and β). However, the PASKIN kinase domain is even more closely related to proto-oncogene serine/threonine-protein kinases 1, 2 and 3 (PIM1, 2 and 3) (Schläfli et al. 2009). Similar to PIM1 and unlike AMPKs, PASKIN kinase domain activity is independent of activation loop phosphorylation (Kikani et al. 2010). In a recent study, we investigated PASKIN kinase activity in more detail. We determined the kinase target sequence for PASKIN with the 30 candidates most strongly phosphorylated by PASKIN on a microarray of 1176 peptides (Schläfli et al. 2011). The identified target sequence consensus for PASKIN is

similar to the target sequence of protein kinase (PK) A and C and has a strong tendency for an arginine residue at position -3. This was also found to be an important characteristic of PASKIN target sequences in a combinatorial peptide library screen (Kikani et al. 2010). In addition to the previously known targets, we identified new target peptides derived from the ribosomal protein S6, phosphorylase kinase b and 6-phosphofructo-2-kinase / fructose 2,6-bisphosphatase. Additionally, glycogen synthase, the transcription factor pancreatic duodenal homeobox-1 (PDX-1) were found to be phosphorylated by PASKIN (Schläfli et al. 2011). However, mammalian UGP1 is probably not phosphorylated by PASKIN (Hao and Rutter 2008). Still, PASKIN might be involved in storage carbohydrate partitioning since, at least *in vitro*, glycogen synthesis is controlled by PASKIN. Human PASKIN was shown to phosphorylate the rabbit glycogen synthase at Ser-640. This phosphorylation led to a decreased activity of the glycogen synthase. The inhibiting phosphorylation of glycogen synthase itself was inhibited by high glycogen levels. Unexpectedly, a deletion of the PASKIN N-terminus, including the PAS domain and the piece between the kinase and the PAS domain abolished glycogen synthase phosphorylation. Deletion of only the PAS domain resulted in the expected increase of target phosphorylation. Interestingly, activity was still regulated by glycogen independently of the PAS domain, suggesting that the PAS domain of PASKIN is not needed for glycogen regulation of target phosphorylation (Wilson et al. 2005).

We found that PASKIN activity is also regulated by phospholipids *in vitro*. We showed phospholipids to bind and increase autophosphorylation. Among the investigated phospholipids, monophosphorylated phosphoinositols (PtdIns) showed the strongest affinity to PASKIN and the highest ability to induce PASKIN autophosphorylation. Therefore, PtdIns-monophosphates represent the first endogenous ligand for PASKIN. Unexpectedly, binding of PtdIns-monophosphates occurs in the kinase domain rather than in the PAS-domain (Schläfli et al. 2011). These data suggest that PtdIns-monophosphates are not the previously proposed but still unknown endogenous PAS domain ligand. The finding that PtdIns induced autophosphorylation did not result in increased target phosphorylation is an additional challenge for the canonical model for PASKIN kinase activity regulation.

3.5 The mammalian PAS kinase PASKIN as a metabolic sensor

3.5.1 *In vitro* evidence for PASKIN acting as a metabolic sensor

It is tempting to make conclusions based on the functional similarities of the *in vitro* kinase targets found for mammalian PASKIN and the ones found for the yeast orthologs Psk1 and Psk2. However, some of the results supporting this idea are very weak and sometimes even contradictory. In the mouse insulinoma cell line Min6 and isolated rat islets, glucose increased Paskin mRNA and protein expression and even kinase activity. It was suggested that Paskin is responsible for the glucose induced expression of preproinsulin (*Pre-proins)* and Pdx-1 mRNA (da Silva Xavier et al. 2004). In line with this, the same group suggested that the phosphorylation of Pdx-1 by Paskin regulates nuclear import of Pdx-1 in pancreatic β-cells (An 2006). However, the mechanism suggested for this was not conclusive. Although An and co-workers proposed a direct phosphorylation of Pdx-1 by Paskin, another group suggested that Paskin is influencing Pdx-1 stability via regulating glycogen synthase kinase 3 beta (Gsk3β). This is based on the observation that PASKIN phosphorylates Gsk3β *in vitro* and that PASKIN overexpression is stabilizing PDX-1 protein levels and therefore mimics the effects of high glucose (Semache et al. 2013). However, Semache and co-workers failed to provide proof that the effect of PASKIN overexpression on PDX-1 stability is by Gsk3β phosphorylation. Although the mechanisms behind are still unclear, it suggests that PASKIN is involved in glucose regulation of insulin expression via PDX-1. Another study showed a slight decrease of PASKIN expression in human islets isolated from patients with type 2 diabetes compared to healthy donors (da Silva Xavier et al. 2011). Further *in vitro* data was presented suggesting an additional role for PASKIN in α-cells. Based on this it has been proposed that PASKIN acts as a glucose sensor in β-cells and α-cells. In β-cells, PASKIN is supposed to increase insulin secretion whereas in α-cells PASKIN inhibits glucagon secretion via inhibition of PRKAA (AMPKalpha2) expression and glucagon expression (da Silva Xavier et al. 2011). However, the human PASKIN mutation pG1117E identified in a family with occurrence of inherited diabetes showed increased target phosphorylation *in vitro*. Infection of rat pancreatic islets with this mutation results in increased basal insulin secretion and expression of *Pre-proins 2* and *Pdx-1*. The induction of insulin secretion by glucose is decreased. However, expression of PASKIN pG1117E does not affect glucagon secretion in rat pancreatic islets (Semplici et al. 2011). Semplici and colleagues fail to provide a conclusive mechanism behind these partially contradicting results.

Interestingly, glucose induced increase in Paskin mRNA in Min6 cells and in isolated rat islets was shown to be inhibited by the presence of palmitate. In addition, overexpression of Paskin reduced the inhibitory effect of palmitate on the glucose induced expression of insulin. The inhibitory effect of palmitate was also reduced by inhibiting the activity of the extracellular-signal-regulated kinases (ERK1 and 2) (Fontés et al. 2009). This suggests that Paskin might be protective against the detrimental effects of increased levels of glucose combined with increased levels of fatty acids observed *in vitro*. This oberservation is usually referred to as the glucolipodtoxicity hypothesis (Poitout and Robertson 2008). However, the regulation of Paskin by glucose and the influence of Paskin activity on the hormone household regulating the glucose metabolism are difficult to reproduce (Borter et al. 2007). This might be due to different effects on PASKIN expression in different contexts. In β- and α-cells from the pancreas glucose might increase PASKIN expression and activity (da Silva Xavier et al. 2004; da Silva Xavier et al. 2011). In explants derived from rat ventromedial and lateral hypothalamus, Paskin protein and mRNA levels are reduced by increased glucose concentrations. Interestingly, glucagon-like peptide 1 (GLP-1) seems to weaken the glucose-induced reduction of Paskin levels in explants from the lateral hypothalamus but not in the ventromedial hypothalamus (Hurtado-Carneiro et al. 2013). This suggests that Paskin, like in the pancreas, could be involved in glucose sensing in the hypothalamus. But, once more regulation of Paskin expression is very context dependent. The neuroblastoma cell line N2A show the same glucose-induced effect on Paskin expression as observed in the lateral hypothalamus (Hurtado-Carneiro et al. 2013). Also in N2A cells this effect is impaired by Glp-1. Interestingly, Paskin knockdown in N2A cells seems to influence mRNA and protein levels and the activity of other metabolic sensors in response to glucose changes and GLP-1 (Hurtado-Carneiro et al. 2013). Based on these observations, Hurtado-Carneiro and colleagues suggested that Paskin might be involved in hypothalamic food intake regulation but no mechanism has been proposed.

3.5.2 The *Paskin* knockout mouse

A more systemic idea of the role of PASKIN as a metabolic sensor was gained by the investigation of the *Paskin* knockout mouse. In mouse, as in human, Paskin consists of 18 exons (Hofer et al. 2001). In the *Paskin* knockout mouse, the exons 10-14 forming the kinase domain were replaced by an En2-IRES-βGeo cassette (Katschinski et al. 2003).

Paskin knockout mice seem to undergo a normal embryonic development. Additionally, normal litter size suggests that *Paskin* knockout mice show no impairment of fertility.

Furthermore, female and male *Paskin* knockout mice show a normal gain of body weight and viability under standard animal husbandry conditions (Katschinski et al. 2003). Although *in vitro* data was published suggesting Paskin is involved in insulin secretion, pancreatic islet β-cells derived from *Paskin* knockout mice show no impairments of induction of insulin expression or insulin secretion by glucose (da Silva Xavier et al. 2004; Borter et al. 2007). Additionally, measurement of blood glucose concentrations and glucose tolerance tests revealed no differences between wildtype and *Paskin* knockout mice of age 6-8 weeks or 14-16 weeks. The lack of any phenotype related to impaired glucose measurement and insulin expression and secretion shows that the *in vitro* data concerning a role of PASKIN in regulation insulin expression and secretion should be approached very cautiously. However, Hao and colleagues found that *Paskin* knockout mice seem to be partially protected from the adverse effects of a high fat diet (Hao et al. 2007). Solely under these conditions, *Paskin* knockout mice showed increased sensitivity to insulin and a better glucose tolerance than wildtype mice (Hao et al. 2007). Furthermore, *Paskin* knockout mice gained less body weight when fed with high fat diet. Hao and co-workers found *Paskin* knockout mice showed a slightly increased metabolic rate (Hao et al. 2007). We also observed reduced gain of body weight of *Paskin* knockout mice compared to wildtype under high fat diet. However the observed differences were not significant. Interestingly, this effect disappeared when the fat content of the diet was increased even more from 45% to 60% (Schläfli et al. 2009). These partially contradictory data are not easy to interpret. Although, data from the rat hypothalamus, reported by Hurtado-Carneiro and colleagues, suggests a difference in the hunger/satiety regulation, at least the protective effect of a *Paskin* knockout from obesity cannot be explained by an impaired food intake because we carefully controlled for any changes in the amount of food consumed (unpublished data). Recently, a study suggested that *Paskin* knockout mice have an impaired reaction to exendin-4, a structural homolog of GLP-1 used in the treatment of diabetes mellitus type 2 due to its longer half live *in vivo*. This was explained by interference of loss of Paskin with Ampk and S6k activity (personal communication with Hurtado-Carneiro).

Additionally, it was reported that female *Paskin* knockout mice react to acute hypoxia with an increased ventilatory response. This reaction was not observed in male knockout animals. Similar gender differences occurred in chronic hypoxia. However, no mechanism has been proposed explaining the differences in the ventilatory response between female and male knockout mice (Soliz et al. 2008).

3.6 The PASKIN tissue specific expression pattern

As delineated above the data about the the biological function of PASKIN is difficult to interpret. There are many open questions not only about the function but also about the upstream regulation of PASKIN. This might be because PASKIN has different function depending on the tissue it is expressed. A closer investigation of the transcriptional regulation of PASKIN might provide new insights in the processes PASKIN is involved.

Northern blot analysis of mouse tissue showed that Paskin mRNA expression is in general very low. As the only exception, testis shows a very high expression of Paskin mRNA followed by thymus (Katschinski et al. 2003). These results were later confirmed by RT-qPCR data (Borter et al. 2007). Paskin mRNA is highly expressed in testis followed by bone marrow, brain and thymus. Only very weak expression of Paskin was found in pancreas. This pattern of expression of Paskin provides little support for a role in pancreatic α- or β-cells (Borter et al. 2007). Furthermore, β-galactosidase, expressed under the endogenous *Paskin* promoter in the *Paskin* knockout mice, provides elegant tool to investigate Paskin expression *in vivo* (Katschinski et al. 2003). X-gal staining of tissues derived from *Paskin* knockout mice revealed that Paskin is highly expressed in testis, more specifically in the seminiferous tubules. β-galactosidase activity and in situ hybridisation data suggest an increase of Paskin expression and protein levels during spermatogenesis in spermatocytes, spermatids and spermatozoa (Katschinski et al. 2003). Immunohistochemistry, showed that PASKIN is present in the cytoplasm and in the nucleus in spermatogonia and spermatocytes (Eckhardt et al. 2007). Immunofluorescence staining for PASKIN showed no nuclear localisation in ejaculated sperm cells. Here PASKIN is localized to the midpiece of the tail. Despite the high expression in testis and sperm cells, the maturation of sperm cells and sperm motility are not affected in *Paskin* knockout animals (Katschinski et al. 2003; Eckhardt et al. 2007).

This leaves only the interesting observation that Paskin mRNA levels are high in tissues of the immune system. A putative role of PASKIN in the immune system has not been investigated until now. A goal of this thesis was to gain more insights about the interesting mRNA expression pattern in the immune system.

3.7 The immune system and its components

Defence against invading pathogens is crucial for every living organism. In higher eukaryotes this is mainly achieved by the immune system. Dysfunctions of the immune system leave the organism vulnerable to external attacks and infections. On the other side, disorders in the regulation of the immune system lead to inflammatory diseases, autoimmune diseases and cancer. Apart from the testis, Paskin mRNA expression is highest in organs associated with the immune system like the thymus and bone marrow (Katschinski et al. 2003; Borter et al. 2007). This implicates a putative role for PASKIN in immune cells and the immune response. The immune response is divided in an adaptive and an innate response. Both are based on leucocytes. All leucocytes are derived from hemapoetic stem cells in the bone marrow. The hemapoetic stem cell is able to divide and differentiate into lymphatic- and myeloid stem cells. The lymphatic and myeloid stem cells further differentiate into a series of lymphatic or myeloid cell types. To track the different cell types, a system of surface proteins are used as markers, called cluster of differentiation (CD).

3.7.1 Myeloid lineage

The myeloid lineage of the immune system includes the monocytes and the polymorphonuclear leukocytes or granulocytes. Polymorphonuclear cells or granulocytes are further divided into neutrophil, basophil and eosinophil granulocytes. Their development takes place mainly in the bone marrow. Monocytes and granulocytes form together the major part of the adaptive immune system. Macrophages are phagocytotic cells. Some macrophages, called resident macrophages, migrate in the tissue until they detect chemotactic factors released at the side of pathogen invasion. Pathogen specific substances are detected by macrophages by a series of receptors. For example lipopolysaccharides (LPS), a component of cell walls of gram-negative bacteria, is detected by a receptor composed by CD14 together with toll like receptor 4 (TLR4). After phagocyting a pathogen macrophages become professional antigen presenting cells (APC). Additionally, they are able to recruit neutrophils, among other granulocytes, to the site of infection via secretion of cytokines. This is the start of an inflammation. Similar to macrophages neutrophils have phagocytotic activity. In a second phase blood monocytes are recruited to the site of inflammation. A complex signalling system of cytokines mediates the inflammation and leads to systemic changes, including e.g. increased release of granulocytes into the blood stream and fever.

3.7.2 Lymphoid lineage

The lymphatic stem cell further specialises into mononuclear T- or B-cells and natural killer cells. T- and B-cells are providing the adaptive immune response. Both T- and B-cells are induced by activation by a presented antigen. Both cell lines undergo a phase of proliferation and maturation before being able to be activated.

From the bone marrow T-cells migrate to the thymus where they mature before entering the circulation of the blood and lymphatic system. The maturation phase acts as a fail-safe system to ensure functionality and prevent auto-reactivity. Mature T cells are divided into two main classes distinguishable by the expression of CD8 or CD4. Upon activation they differentiate in so called effector cells. CD8 differentiate in cytotoxic T cells and CD4 differentiate in T-helper cells. Both T-cell subsets express CD3 that is associated with the T cell receptor, a heterodimeric immunoglobulin. The CD3-T-cell receptor complex is activated by the recognition of a specific antigen. The antigen can be presented by APC or by infected cells via the major histocompatibility complex 1 (MHC1) and is then recognised by CD8 positive cytotoxic T-cells. CD4 positive helper T-cells recognise antigens presented by APC via MHC2. Stimulation of the T-cell-CD3 receptor complex and activation of its downstream signalling cascade is not enough to trigger proliferation and differentiation into effector cells. CD3, the T cell receptor and CD8 or CD4, respectively, are part of a higher structure called the immunological synapse. A professional antigen presenting cell (APC) and the receiving T-cell are forming a synapse-like connection. On the T-cell site the immunological synapse is composed of three so called supra-molecular activation complexes (SMACs) arranged in three rings. Antigen recognition takes place in the center area formed by cSMAC including CD3, the T-cell receptor and CD28. Stimulation of CD28 is important for a normal T cell response. Activation of CD28 is needed for proper interleukin2 (IL-2) secretion (Lucas et al. 1995). CD28 stimulation activates phosphoinositide 3-kinase (PI3K) (Pages et al. 1994). This function is shared with CD2 another important costimulatory receptor (Kivens et al. 1998). Both CD28 and CD2 induce also PTEN suggesting a negative feedback loop (Schmidt-Weber et al. 2002). Additionally, stimulation of CD28 induces recruitment of more receptors to the synaptic site. CD28 recognises CD80 and CD86 on the APC. CD2 binds to the cell adhesion molecule lymphocyte function-associated antigen-3 (LFA-3) or (CD58) (Selvaraj et al. 1987) The center area is surrounded by a ring built by the peripheral pSMAC. The proteins of the peripheral ring are involved in cell-cell adhesion. An additional distal ring built by dSMAC

contains proteins like CD43 and CD45 (Huppa and Davis 2003). Recognition of a presented antigen goes in line with dynamic restructuring of the cytoskeleton and repositioning of lipid rafts to the immunological synapse. These cytoskeleton rearrangements are depending on ERM family protein phosphorylation status (Roumier et al. 2001). The successful T-cell activation leads to the secretion of IL-2. Autostimulation of T-cells by IL-2 leads to clonal expansion and differentiation to effector cells. CD8 positive cytotoxic T-cells proliferate and head out to kill infected cells presenting the activating antigen forming again an immunological synapse. The CD4 positive T helper cell forms yet another immunological synapse. This time with a naive B-cell specific for the same antigen that activated the T-cell. B-cells can also be activated by cytokines secreted by T-cells like IL-2.

The maturation process of B-cells takes place in the bone marrow. B-cells express the B cell receptor (BCR) an immunoglobulin specific to an antigen anchored to the cell surface. B-cells expressing a BCR recognizing an endogenous antigen are eliminated during their maturation phase in the bone marrow. Mature B-cells leave the bone marrow and migrate to the lymph nodes. Activated by contact with their specific antigen B-cells proliferate and differentiate into antibody producing plasma cells. Successful activation includes costimulation by a T-helper cell recognising the same antigen. The threshold for activation of naive B-cells is decreased by stimulation of the surface protein CD19 an important marker for B-cells. B-cells can also be activated T-cell independently by endotoxins like LPS via CD14 and TLR4 (Poltorak et al. 1998; Rhee and Hwang 2000). Stimulation with LPS induces the secretion of pro-inflammatory cytokines like TNF-α.

Although derived from a lymphoid precursor cell natural killer cells (NK-cells) are not antigen specific and therefore belong to the innate immune system in contrast to T- and B-cells. Various developmental states of NK-cells are found in the bone marrow, spleen, thymus and the lymph nodes (Di Santo 2006). NK-cells express so called killer-cell immunoglobulin-like receptors (KIR) recognising different classes of MHCs. Binding of MHC to KIR inhibits the killing activity of NK-cells. This allows NK cells to recognize cells with altered MHC expression. This has been observed in tumour cells and infected cells (Vilches and Parham 2002). NK-cells express CD56, the neural cell adhesion molecule NCAM. The cytotoxic activity of NK-cells is increased by IL-2 and interferones (Henney et al. 1981). Natural killer cells together with macrophages, neutrophils, eosinophils and mast cells express receptors that recognise the FC-region of antibodies like FCγRIII or CD16.

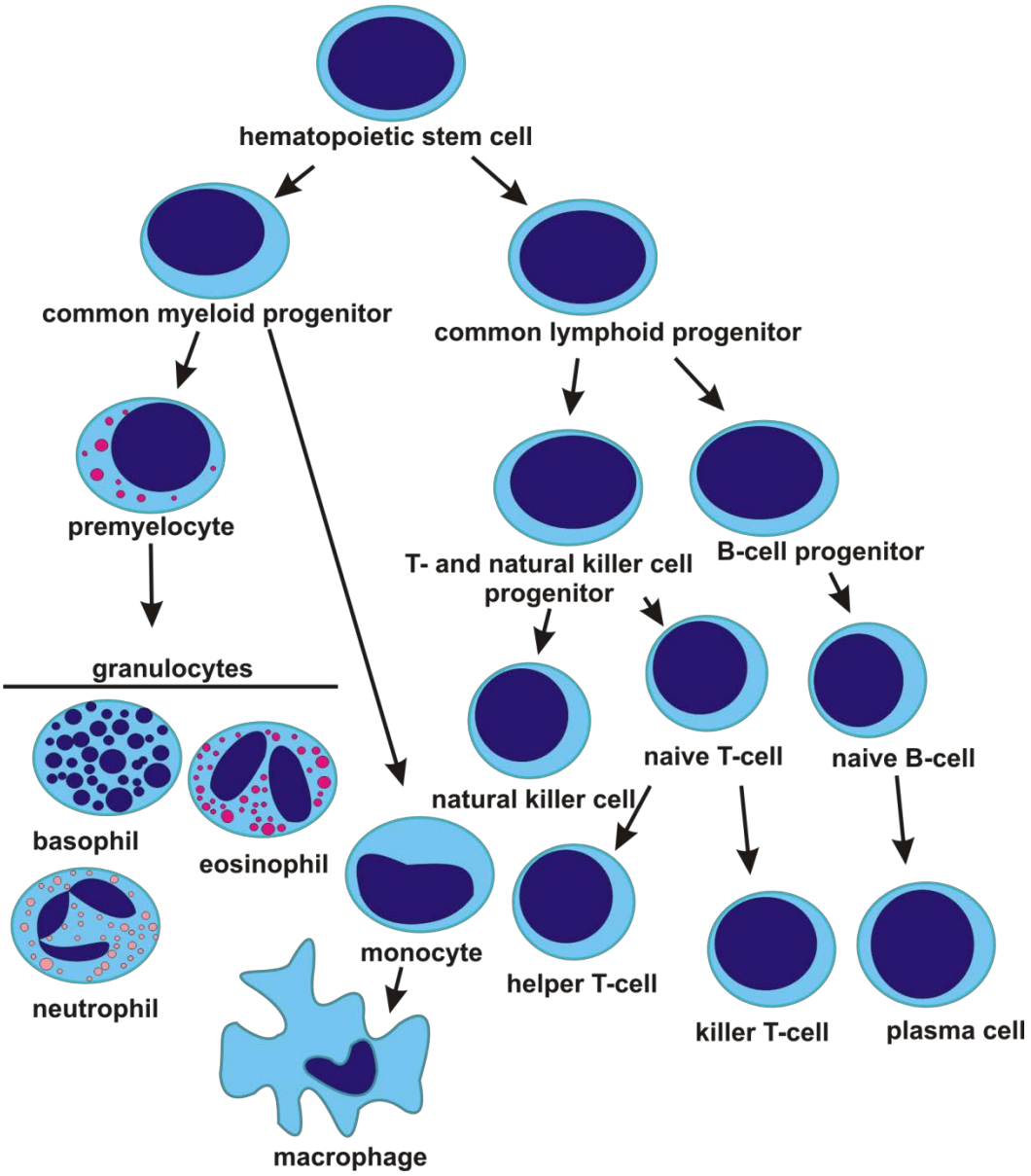

Fig. 3. Overview of hematopoiesis. Depicted are the lymphoid and myeloid lineage of hematopoiesis as described in the text. The erythrocyte and megacaryocyte branch as well as dendritic cells are not shown. Consult text for details.

3.7.3 *In vitro* models for immune cells

Myeloid differentiation can be studied *in vitro* with the cell line HL-60. The cell line was isolated from a 36 year old woman with acute promyelocytic leukemia. The isolated cells are positive for myeloid lineage specific markers like myeloperoxidase (MPO). However, they are negative for alkaline phosphatase which is characteristic for normal neutrophils. In short, HL-60 cells seem to exhibit a promyelocytic character. However, it was observed that 5% - 10% of the cultured HL-60 cells undergo spontaneous differentiation in to more mature granulocytes (Gallagher et al. 1979). The fraction of differentiated HL-60 cells can be

increased to up to 90% with dimethyl sulfoxide (DMSO) and retinoic acid (Breitman et al. 1980).

The cell line Jurkat is a widely used model for T-cell activation and T-cell receptor signalling (Abraham 2000). The Jurkat cell line was isolated from a 14 year old boy with acute lymphoblastic leukemia. The Jurkat cell line shows expression of CD2, complement and FC-receptor and other T-cell characteristics (Schneider et al. 1977). Most importantly Jurkat cells express the T-cell receptor and CD3. An important characteristic of the Jurkat cell line is the ability to produce substantial quantities of IL-2 when successfully stimulated with the lectin phytohemagglutinin (PHA) (Gillis and Watson 1980).

An even more important source for *in vitro* studies of human immune cells than leukemic cell lines is the isolation of primary cells from whole blood or from buffy coats. The peripheral mononuclear cells (PBMCs) are isolated from granulocytes and erythrocytes by density gradient centrifugation. After being separated from the PBMCs granulocytes can be purified simply by lysis of erythrocytes. The PBMCs can be further separated into subset by positive or negative affinity separation with antibodies against subset specific markers or by flow cytometry. The different subsets can be selectively activated by a series of immunostimulants.

In vitro, the necessary costimulation required to successfully activate T-cells can be achieved with a mixture of antibodies against CD2, CD28 and CD3. Due to the partial redundancy of CD2 and CD28, mixtures of anti CD3 with CD28 or CD2 can be sufficient. The activation of the T-cells results in IL-2 secretion what further enhances T-cell proliferation. IL-2 also influences proliferation of B-cells (Mingari et al. 1984). Furthermore, B-cells can be activated in vitro by unmethylated CpG oligonucleotides. This effect is enhanced by costimulation with antibodies against IgM (Krieg et al. 1995). The unmethylated C in CpGs are typical for bacterial DNA and are recognised by the human toll-like receptor 9 (TLR9) (Bauer et al. 2001). Additionally, B-cells are activated by LPS over TLR4 as mentioned before (Poltorak et al. 1998; Rhee and Hwang 2000). Toll-like receptor stimulation of PBMC will also activate monocytes and macrophages.

Immunosuppressants are used to suppress an immune response. A T-cell selective immunosuppressant is cyclosporin A. Cyclosporin is a fungal polypeptide binding to cyclophilin. This binding inhibits calcineurin and thereby blocks the Ca^{2+} release induced by T-cell activation. The increase of intracellular Ca^{2+} is needed for the activation of the nuclear factor of activated T-cells (NFAT). NFAT is a transcription factor involved in IL-2

expression. Dexamethason (9-fluor-16α-methylprednisolone) is another immunosuppressant widely used. Dexamethason is a synthetical corticosteroid. It is an agonist of the broadly expressed glucocorticoid receptor and therefore much less specific than cyclosporin.

This provides a multitude of possibilities to investigate the regulation of PASKIN mRNA levels in immune cells. The high PASKIN mRNA levels in tissues of the immune system might be due to increased PASKIN expression in immune cells predominantly present in these tissues. It is therefore reasonable to determine PASKIN mRNA levels in leukocyte cell lines and primary leukocytes. If this hypothesis proofs true it is of particular importance to identify the immune cell type that has the highest PASKIN mRNA levels. Stimulation with the described pro-inflammatory stimuli and immune suppressant would provide further insight in PASKIN expression regulation and function. Since the hypothesis of a role for PASKIN in immune response is based on the expression of PASKIN mRNA. These investigations could be supplemented with the closer examination of the genomic regulatory elements responsible of this immune system specific expression regulation

3.8 Genomic organisation of the *PASKIN* locus

The human *PASKIN* gene is localized at position (chr2:242,045,514-242,088,919) UCSC hg19 on the reverse strand. Only approximately 1 kilobase apart on the opposite strand at position (chr2:242,089,902-242,106,592) the protein phosphatase 1 regulatory subunit 7 (*PPP1R7*) is localised. This constellation is conserved in mouse (Hofer et al. 2001). The close proximity of these two genes could indicate a shared promoter and therefore a transcriptional coregulation. Indeed, in mouse it has been shown that Ppp1r7 mRNA is expressed in the same organs as Paskin. Particularly, the high levels of Ppp1r7 protein in the testis is striking (Katschinski et al. 2003). The protein levels of Ppp1R7 in testis are independent of *Paskin* genotype. However, mRNA expression detected by Northern blot showed a slight increase of Ppp1R7 in tissue derived from *Paskin* knockout mice (Katschinski et al. 2003). This could be explained by an increased bidirectional activity of the *Paskin-Ppp1r7* promoter locus in an attempt to compensate for a lack of Paskin. Indeed, Hurtado-Carneiro and colleagues could observe increased expression of Paskin-βgeo fusion-mRNA in knockout mice by qPCR with a primer pair specific for the PAS region of the Paskin-βgeo fusion-mRNA (personal communication). Genes regulated by bidirectional promoters have been found to be often involved in DNA damage repair or chromosome organisation (Adachi and Lieber 2002)

(Trinklein et al. 2004) (Wakano et al. 2012). Interestingly, pairs of genes sharing a bidirectional promoter are more likely to be involved in the same biological processes (Li et al. 2006). Considering the PASKIN is a serine/threonine kinase and PPP1R is a regulatory subunit of a phosphatase the possibility of a co-regulation and even a functional connection is tempting.

3.8.1 The protein phosphatase 1 regulatory subunit 7

PPP1R7 is the mammalian ortholog of the essential yeast gene *Sds22*. Disruption of *Sds22* results in an arrest in midmitosis (Ohkura and Yanagida 1991). In yeast, Sds22 interacts with Glc7, the yeast ortholog of PP1, the catalytic subunit of the serine/threonine phosphatase 1. Overexpression of Sds22 is able to suppress a temperature sensitive *Glc7* mutant strain (MacKelvie et al. 1995). The protein Glc7 has been shown to be required for glycogen accumulation. Additionally, Glc7 is important for cell growth and division, cell wall integrity and actin localisation (Ohkura and Yanagida 1991). Interestingly, the role of Sds22 is rather to direct and target the phosphatase activity of Glc7 to specific target proteins involved in various processes than regulate the overall activity of Glc7 (Pedelini et al. 2007). This explains the conflicting reports of activation and inhibition of Glc7 activity by Sds22.

In *Drosophila melanogaster* a reduction of Sds22 results in changes of cell polarity. This goes in line with a reduction of the F-actin/G-actin ratio. This was explained by increased phosphorylation of ERM family proteins caused by the loss of Sds22. The ability of Sds22 to reduce ERM protein phosphorylation is conserved in human (Grusche et al. 2009). The ERM protein family is named after their main members ezrin, radixin and moesin. The ERM proteins are participating in organizing the cell cortex. Managing interactions of the cell membrane with the cytoskeleton they are involved in series of signalling processes (Neisch and Fehon 2011).

Another process targeted by PP1 in concert with PPP1R7 that involves the cytoskeleton is the spindle checkpoint. PPP1R7 is needed for localisation of PP1 to the kinetochore. By dephosphorylation of aurora B, among a series of other targets at the kinetochore, PP1 together with PPP1R7 counteracts aurora B and therefore stabilizes the attachment of microtubules and allows mitosis to proceed. (Liu et al. 2010; Posch et al. 2010; Wurzenberger et al. 2012).

The involvement of the Glc-7 the target of Sds-22 in glycogen metabolism and the regulation of cell wall integrity provides the first indications that PASKIN and PPP1R/ might have a closer functional connection than presumed so far. Interestingly, the *Drososphila* Paskin has been recently associated with the spindle checkpoint. The role of PPP1R7 in this biological process might be therefore no coincidence.

3.9 The spindle assembly checkpoint and PASKIN

A recent publication also brings Paskin in relation to the spindle checkpoint (Shaukat et al. 2012). The group of Stephen Gregory showed that a double knockdown of Paskin and Mad2 in *Drosophila* shows synthetic lethality. MAD2 is an important part of the spindle assembly checkpoint (SAC), which prevents cell cycle progression until all chromosomes are attached to the spindle apparatus correctly. Accurate duplication of the genetic information during cell division is important to suppress accumulation of mutations. In eukaryotes it is crucial not only that all the information is copied but also that all genomic material is distributed equally to the two daughter cells. Therefore, unattached kinetochores lead to the formation of the mitotic checkpoint complex (MCC). The main function of MCC is to inhibit the anaphase promoting complex, also called cyclosome (APC/C) (Sudakin et al. 2001). The APC/C acts as an E3 ubiquitin ligase. Several proteins to be marked for proteolytic degradation are recognized by APC/C (Pines 2011). Among them securin and cyclin B1 are the most important. Securin binds to separase preventing it from cleaving the SCC1 subunit of the cohesion ring. The cohesin ring is keeping the sister chromatides together preventing them from separation (Nasmyth and Haering 2009). Cyclin B1 is required for cyclin-dependent kinase 1 activity (Musacchio and Salmon 2007). The activation of APC/C induced by the lack of an unattached kinetochore signal therefore leads to the degradation of securin and cyclin B1. This allows the release of separase and the inactivation of CDK1 and ultimately the separation of the sister chromatides and mitotic exit (Lara-Gonzalez et al. 2012). As a part of the MCC complex, mitotic arrest deficiency 2 (MAD2) has a key role in regulating APC/C activity. MAD2 is crucial for the detection of the open kinetochore signal. MAD2 exists in two conformations. In the so called open conformation (o-MAD2), MAD2 is able to bind to MAD1. By changing to the closed conformation (c-MAD2), MAD1 is trapped within c-MAD2. The resulting MAD1-c-MAD2 complex is recruited to the open kinetochore (Shah et al. 2004). There, more MAD2 is recruited to the bound MAD1-c-MAD2 complex. Only the o-

MAD2 bound to MAD1-c-MAD2 is able to bind CDC20 thereby changing to c-MAD2 and trapping CDC20 (De Antoni et al. 2005). This CDC20-c-MAD2 complex recruits another subcomplex consisting of MAD3/BUBR1-BUB3 to form the MCC (Musacchio and Salmon 2007).

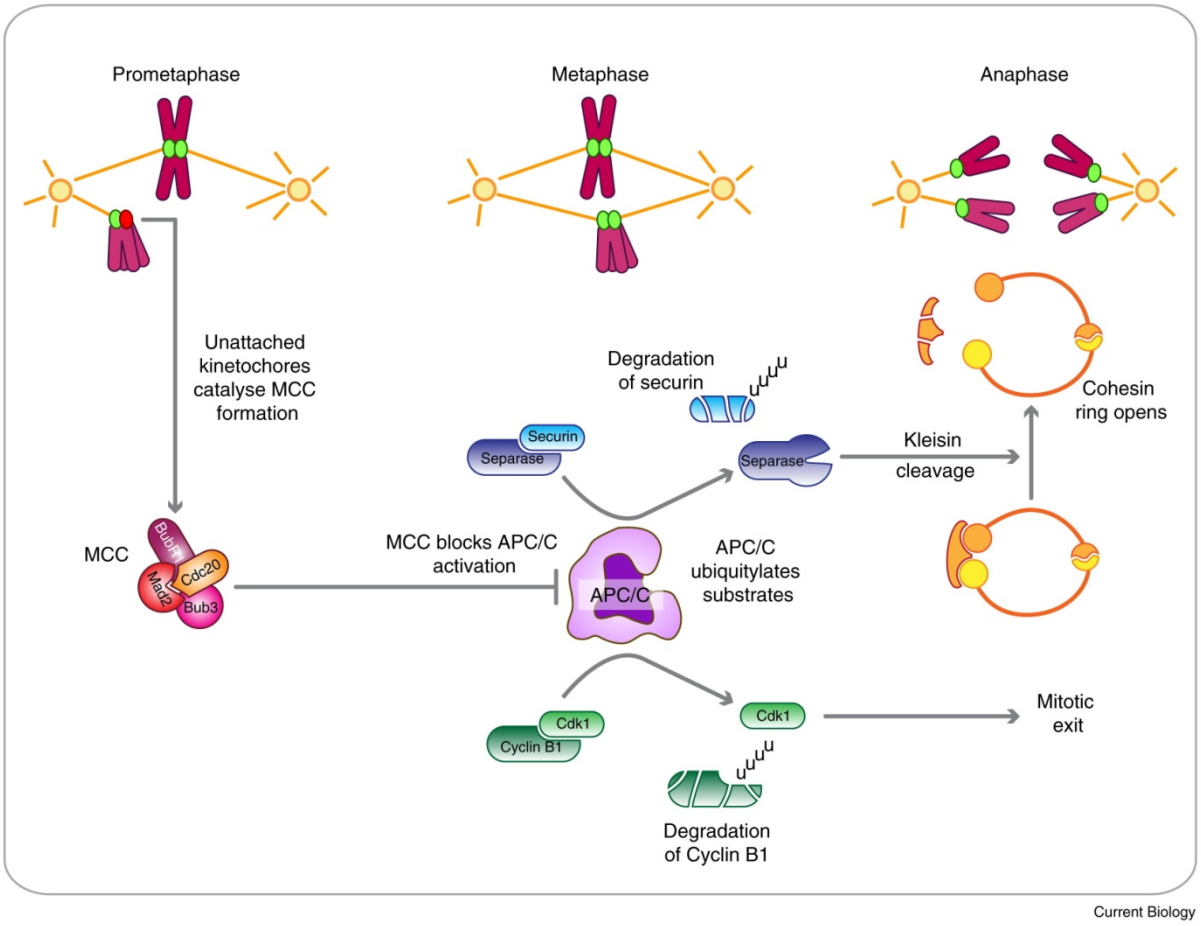

Fig. 4. Overview of the spindle assembly checkpoint. Unnattached kinetochores lead to the formation of the mitotic checkpoint complex (MCC). The main role of the MCC is to inhibit the function of the anaphase promoting complex/cyclosome (APC/C). The inhibition of the ubiquitination of securin and cyclin B1 prevents kleisin cleavage and mitotic exit and therefore progression in the cell cycle (Lara-Gonzalez et al. 2012).

Impairment of the spindle assembly checkpoint among others is one cause for chromosomal instability (CIN). The abnormal number and structures of chromosomes resulting from CIN are associated with prognosis of a poor clinical outcome. CIN favours tumor evolution and therefore leads to increased metastasis and increased drug resistance. CIN can be induced by depleting MAD2 (Michel et al. 2001). The group of Stephen Gregory used a CIN model in *Drosophila* in a synthetic lethality screen for phosphatases and kinases triggering apoptosis in CIN cells (Shaukat et al. 2012). CIN was induced by knockdown of Mad2 by RNAi. Heterozygous Mad2 dsRNA expressing females were crossed with male homozygous for the

dsRNA allele of the investigated kinase. The resulting offspring were either CIN by Mad2 knockdown or not but in any case they expressed the kinase dsRNA. Thus a shift in the offspring ratio indicates increase of CIN specific lethality by the candidate gene. The *Drosophila* kinome of 397 genes was screened. Interestingly, Paskin was among the candidate kinases. The knockdown of Paskin showed a strong negative effect on viability in combination with a knockdown of Mad2. The ratio of Paskin dsRNA expressing offspring to Paskin and Mad2 dsRNA expressing offspring was 13.25. Furthermore, double knockdown of Paskin and Mad2 in wings resulted in tissue loss. Apparently, this tissue loss was caused by p53 dependent apoptosis. Additionally, immunostaining against γH2AX suggest increased DNA damage in Mad2, Paskin double knockdown wing discs compared to Paskin knockdown or Mad2, lacZ double knock downs. Importantly, Paskin knockdown alone showed no increase in anaphase defects suggesting no necessity for Paskin in chromosomal segregation. Additionally, similar amounts of cell death were seen in BubR1, Paskin double knockdown wing discs (Shaukat et al. 2012). These findings suggest that Paskin suppresses DNA damage caused cell death in a background with a weakened spindle assembly checkpoint in *Drososphila*. Till now no research in a mammalian system on a putative role of PASKIN in the context of the spindle assembly checkpoint has been published. Homozygous knockout of *Mad2* in mice results in death during early embryonic stages (Dobles et al. 2000). However, heterozygous *Mad2* mutant mice cells with reduced Mad2 levels show increased occurrence of premature sister chromatide separation and aneuploidy. Mice with a haplo-insufficiency for Mad2 are viable but develop lymphomas and lung tumours at a higher frequency than wildtype mice (Michel et al. 2001). *Paskin* knockout mice develop normally and no increase of tumour frequency has been reported (Katschinski et al. 2003). Since the experiments in *Drosophila* show that only the double-insufficiency for Paskin and Mad2 show a strong phenotype, it would be of great interest to investigate a crossing of the *Paskin* and the *Mad2* knockout mice.

3.10 Goals of the thesis

As outlined above, PASKIN function is unclear. Focussing on a putative role of PASKIN in the pancreas did not result in new insights for years. Although the idea of a role in energy metabolism is quite obvious considering the function of the yeast orthologs of PASKIN, the evidence for this role in the mammalian system is questionable. Therefore I considered

looking at PASKIN function from another angle. The relatively high Paskin mRNA levels in mouse tissues of the immune system suggested a putative role for Paskin in leukocytes. I aimed to further investigate the expression of PASKIN in leukocytes. I was particularly interested in the regulation of PASKIN mRNA levels during the activation of lymphocytes. An additional goal was to gain more insights in the regulation of PASKIN gene expression and the putative co-regulation of PPP1R7. The potential function connection of PPP1R7 and PASKIN implied by a bidirectional promoter might help to better understand PASKIN. Since the yeast ortholog of PPP1R7 is involved in the spindle checkpoint and PASKIN has been associated with this process in *Drosophila*, the question was raised if the mammalian PASKIN is involved in the spindle checkpoint, as well. Therefore, I aimed next to evaluate a role of PASKIN in the cell cycle control in cell lines and in primary MEFs derived from *Paskin* knockout cells.

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4 MANUSCRIPT I: (UNPUBLISHED)

Regulation of PASKIN levels during leukocyte activation

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Abstract

Expression of the mouse PAS domain kinase Paskin is highly tissue specific. Although, most abundantly expressed in testis, Paskin is not essential for sperm development and mouse fertility. Paskin is also expressed in a substantial amount in the bone marrow, although it does not reach the expression levels of the testis. We could confirm this expression pattern in human tissues. Additionally, we found a high expression in human peripheral blood mononuclear cells (PBMC). Furthermore, expression levels of PASKIN were high in human immune cell lines like HL-60 and Jurkat. Lipopolysaccharide (LPS) and phytohemagglutinin (PHA) stimulation of human and mouse cell lines did not affect PASKIN mRNA levels. Similar results were found by stimulation of isolated CD3 and CD19 positive cells. Whole PBMC cultures stimulated with LPS or anti CD3/CD28/CD2 antibody mix showed no changes in PASKIN mRNA expression. We confirmed these findings on protein levels by FACS analysis. In conclusion, we found that there is no regulation of PASKIN expression in immune cells by immune stimulants or inhibitors suggesting that there is no increased demand for PASKIN in the immediate immune response. However, this does not exclude a role for PASKIN in lymphocytes, since *Paskin* knockout mice showed small differences in B-cell response to LPS.

Introduction

PAS domain-containing proteins act as sensors in various processes. In the root nodule bacterium *Bradyrhizobium japonicum* the PAS domain-containing histidine kinase *FixL* acts as a heme-based oxygen sensor involved in nitrogen fixation (David et al. 1988). We and others identified the PAS domain-containing serine/threonine kinase PASKIN as the eukaryotic homolog of FixL (Hofer et al. 2001; Rutter et al. 2001). PASKIN has been implicated in regulating energy homeostasis (Hao et al. 2007; Schläfli et al. 2009) and this function has been proposed to be conserved from yeast to humans (Hao and Rutter 2008). In yeast, two PASKIN orthologs, Psk1 and Psk2, are activated by non-fermentative carbon sources or cell integrity stress, respectively (Grose et al. 2007). Upon activation, Psk1 and Psk2 phosphorylate the UDP-glucose pyrophosphorylase 1 (Ugp1). It has been suggested that this leads to a conformational change of Ugp1 and subsequently to a relocalisation of Ugp1 to the cell periphery (Grose et al. 2007). This results in a switch from storage carbohydrate production to structural carbohydrates production. In addition to Ugp1, that is involved in glycogen synthesis, three proteins involved in protein synthesis; cap-associated factor 20 (Caf20), eukaryotic translation initiation factor 1A (eIF1A) and Sro9, have been proposed as Psk1 and Psk2 targets (Rutter et al. 2002). However, *Paskin* knockout mice show normal glucose tolerance and insulin sensitivity under standard feeding conditions. Interestingly, *Paskin* knockout mice seem to be partially protected from metabolic syndrome when fed with a high fat diet (Hao et al. 2007; Schläfli et al. 2009). It has been suggested that this effects might be due to a higher metabolic rate in *Paskin* knockout mice. Additionally, PASKIN has been described to have a role in the regulation of insulin and glucagon in β-and α-cells of the islets of Langerhans (da Silva Xavier et al. 2004; da Silva Xavier et al. 2011). Although a role for PASKIN has been proposed in the pancreas, the organ with the by far highest Paskin expression is the testis (Katschinski et al. 2003).

The testis is an organ of high proliferation activity in line with an increased energy demand. Despite the high Paskin expression in testis, *Paskin* knockout mice show no sign of impaired fertility (Katschinski et al. 2003). However, PASKIN has been found to colocalize with its kinase target eEF1A1 in the midpiece of the sperm tail (Eckhardt et al. 2007). Nevertheless, no defect in sperm cell motility has been found in *Paskin* knockout mice (Katschinski et al. 2003). Besides the testis, Paskin is highly expressed in tissues associated with leukocyte development including thymus, bone marrow and to a lesser extent spleen. Activation of leukocytes induces blastogenesis and proliferation, in line with increased glucose utilisation

(Roos and Loos 1973; Culvenor and Weidemann 1976). This metabolic switch might involve PASKIN. Additionally, the activation-induced proliferation goes in line with increased protein synthesis, another process PASKIN might be involved in. This would be an explanation for the high mRNA levels of PASKIN in lymphatic organs.

To investigate a putative role of PASKIN in the activation of leukocytes, we stimulated leukocyte cell lines and primary leukocytes with selected mitogens followed by analysis of PASKIN mRNA and protein expression.

Materials and Methods

Cell culture and stimulation of cell lines

Hela, U2OS, A549, MCF7, MDA-MB-231, MDA-MB-468, Hep3B, HepG2 and FTC-133 were cultured in Dulbecco's modified Eagle's media (Sigma) containing 10% FCS (Invitrogen) and 33.1.1, K-46, HL-60, Jurkat and THP-1 were cultured in RPMI 1640 (Sigma), supplemented with 10% FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin (Sigma), at 37°C. Leukocyte cell lines were stimulated with 1 µg/ml lipopolysaccharide (Sigma) in PBS or 5 µg/ml phytohemagglutinin-L (Sigma) in PBS 16 hours after seeding of 1 x 10^6 cells in 10 ml of medium. Isolated PBMCs were cultured in RPMI with 10% FCS supplemented with amino acids (Sigma). PBMCs were treated with antibody mix consisting of anti-human CD3 (orthoclone OKT3, in house production), anti-human CD28 (clone15E8, in house production) and two clones of anti-human CD2 (clone 6g4 and 4B2, Sanquin), IL-2 100 U/ml (Proleukin, Roche), CpG oligonucleotides 1 µM (Microsynth), 1 µg/ml lipopolysaccharide (Sigma) in PBS, 5 µg/ml phytohemagglutinin-L (Sigma) or dexamethason (Sigma) and cyclosporin A (Sigma).

Isolation of PBMCs

Fresh buffy coat (Blutspende Zürich) was filtered through a 70 µm cell-strainer filter (BD) in two 50 ml tubes. The two 50 ml tubes were filled up with 25 ml PBS, 2 mM EDTA, (pH 7.4) and mixed carefully by inverting. The buffy-PBS mix was added to four 50 ml tubes with a filter above prewarmed Bicoll with a density of 1.077 g/ml (Biochrom AG). The tubes were centrifuged at 800 g for 20 minutes at 20°C with low acceleration and no break. The white interphase and some serum was collected and filtered with a 70 µm filter in four fresh 50 ml Falcon tubes. The tubes were filled up with PBS, 2 mM EDTA, (pH 7.4). The cells were

washed by centrifugation at 780 g for 10 minutes at 8°C. To lyse red blood cells, the pellet was resuspended in sterile H_2O by pipetting up and down for not longer than 60 seconds. The suspension was transferred immediately to two 50 ml tubes containing 45 ml PBS, 2 mM EDTA (pH 7.4). The cells were washed by centrifugation at 200 g for 10 minutes at 8°C and resuspended in 50 ml PBS. To exclude granulocyte contamination, isolated cells were analysed in a KX-21N™ automated Hematology Analyzer (Sysmex). Cells were centrifuged at 300 g for 10 minutes at 8°C and then resuspended in RPMI 1640 supplemented with 10% FCS (Invitrogen) and amino acids (Sigma). Cells were counted in a Neubauer chamber

Cell subset separation

To separate cell subsets from isolated PBMCs the following products (MACS, Miltenyi Biotec) were used:

CD8+ T Cell Isolation Kit II Order no. 130-094-154

CD4+ T Cell Isolation Kit II Order no. 130-091-155

B-Cell Isolation Kit II Order no. 130-091-151

CD16 MicroBeads Order no.130-045-701

CD14 MicroBeads Order no. 130-050-201

PBMCs (1×10^8) isolated from buffy coats were resuspended in 400 µl of MACS-buffer. Subsequently, 100 µl of the cell subset-specific biotin cocktail was added. After mixing samples were incubated for 10 minutes at 8°C. 220 µl MACS Buffer, 80 µl of anti-CD16 micro beads, and 200 µl anti-biotin micro beads was added. In the sample for CD4 positive isolation additional 50 µl anti-CD14 micro beads were added. The mixture was incubated for 15 minutes at 8°C and washed with 9 ml MACS-Buffer followed by spinning down for 10 minutes with 300 g at 8°C. For magnetic separation, an autoMACS™ seperator was used. Purity of the separation was analysed by flow cytometry using an Epics XL CLR/MCL (Beckman Coulter).

Total RNA isolation from cells

Cells were harvested in 2 ml solution D (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol). Total RNA was isolated according to Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Briefly, 800 µl of cell lysate was

transferred to a 2 ml tube and carefully mixed with 100 µl 2 M sodium acetate. 1 ml water-saturated acid phenol was added and carefully mixed. The suspension was mixed with 200 µl chloroform and incubated for 20 minutes on ice. To further separate the phases samples were centrifuged at 12'000 g for 20 minutes at 4°C. The supernatant was transferred to a fresh 2 ml tube and precipitated with 800 µl isopropanol at -20°C for 60 minutes. After centrifugation for 20 minutes at 12'000 g at 4°C pellet was resuspended in 100 µl of Solution D and transferred to a fresh 1.5 ml tube. 100 µl isopropanol was added followed by precipitation for 60 minutes at -20°C. The preciptate was collected by centrifugation with 12'000 g at 4°C for 20 minutes. The pellet was washed with 70% ethanol in diethylpyrocarbonate (DEPC)-treated water and then air dried. The isolated RNA was resuspended in DEPC-treated water.

cDNA generation from human tissue RNA and isolated RNA from cells

One µg RNA from total RNA Master Panel II (Clontech) or isolated as described above, was reverse transcribed with RevertAid first strand cDNA synthesis kit (Thermo Scientific) The reaction was done in 20 µl 1x reaction buffer with final concentrations of 1 mM dNTP, 5 µM random hexamer oligonucleotide, 20 U of ribonuclease inhibitor and 200 U Revert Aid M-MuLV reverse transcriptase. Reaction conditions were 10 minutes at 25°C, followed by 60 minutes at 42°C. Samples were heated for 10 minutes at 70°C. cDNA was diluted 1:10 with RNase/DNase free water. RT-qPCR was performed as previously described (Kaufmann et al. 2013).

Intracellular FACS staining

Cells (3 x 10^6) were used for each experiment. Cells were washed twice with PBS, 2 mM EDTA (pH 7.4) and resuspended in 1 ml PBS, 2 mM EDTA pH 7.4. 1 µl viability dye eFluor450 (eBioscience) was added. Cells were incubated at 4°C for 30 minutes. Subsequently, cells were washed twice with MACS-Buffer (Miltenyi Biotec). Pellets were resuspended in 50 µl MACS-Buffer. 20 µl of human Fc blocking reagent (Miltenyi Biotec) was added followed by incubation for 5 minutes at 4°C. Samples were incubated for 15 minutes with antibodies against surface markers. Samples were washed twice with MAC buffer followed by treatment with Permfix–Cytofix (BD) for 20 minutes. Cells were washed twice with Permwash buffer (BD). The pellet was resuspended in 50 µl of Permwash and incubated on ice for 15 minutes. Then Alexa 488 labelled anti-PASKIN antibody or Alexa 488 labeled IC control was added. Cells were incubated for 60 minutes. After washing with Permwash buffer, the pellet was resuspended in 0.1% paraformaldehyde in PBS. FACS

analysis was performed the following day with a Gallios flow cytometer (Beckman Coulter). Analysis of FACS data was performed with Kaluza software (Beckman Coulter).

Antibody labelling

Anti-PASKIN mouse monoclonal IgG2b antibody (Thermo Scientific, MA1-700) was used for labelling with an Alexa fluor 488 APEX antibody labelling kit according to the manufacturer's advice (Invitrogen). In the same procedure a mouse IgG isotype control (eBioscience) was labelled.

Antibodies used for FACS staining

CD16-PE Clone 3G8 (BD)

CD56-PE Clone N901 (BC)

CD8-ECD Clone SFCI21Thy2D (BC)

CD14 PerCP-Cy5.5 Clone M5E2n (BD)

CD4 PC7 Clone RPA-T4 (Biolegend)

CD3 Alexa647 Clone HIT3a (Biolegend)

CD19 APC-Cy7 Clone HIB19 (Biolegend)

Viability Dye eFluor450 (eBioscience)

Results

Tissue-specific regulation of PASKIN mRNA levels is conserved between mouse and human

Although Paskin is ubiquitously expressed the levels of Paskin mRNA vary substantially in different tissues (Katschinski et al. 2003; Borter et al. 2007). Dot blot analysis of human RNA suggests high levels in testis, thymus and lymph node (Hofer et al. 2001). This was further confirmed by RT-qPCR (Borter et al. 2007). To confirm the pattern of PASKIN tissue specifity is conserved, we measured PASKIN mRNA levels in selected samples from a commercially available collection of human mRNA derived from various tissues. As expected, we found high amounts of PASKIN mRNA in human testis. Additionally, PASKIN mRNA levels were high in thymus. Although 30 times lower than thymus and testis, human brain and spleen samples still showed 10 times higher mRNA levels than samples derived from liver or muscle. Additionally, we measured PASKIN expression in primary peripheral blood mononuclear cells (PBMCs) isolated from buffy coats by density gradient centrifugation. PASKIN mRNA levels in the PBMCs were similar to brain and spleen (Fig. 1A). This suggests that the tissue specific regulation of PASKIN is conserved from mouse to human. Furthermore, the high levels of PASKIN mRNA in PBMCs provides further evidence that high amounts of PASKIN mRNA in mononuclear leukocytes is the reason for the high proportion of PASKIN in mRNA derived from thymus, bone marrow and spleen. The high expression levels of PASKIN in cells and tissues of the immune system suggest a role of PASKIN in leukocytes. To find a suitable model to investigate a putative PASKIN function in immune cells, we screened a series of human cell lines for their PASKIN mRNA levels. RT-qPCR results showed high PASKIN mRNA in human leukocyte cell lines. Especially, HL-60 and Jurkat cells showed PASKIN mRNA levels several times higher than e.g. HeLa or U2OS cells (Fig.1B). This further supports the idea of a role for PASKIN in immune cell lines and confirms our finding in primary cells. Additionally, it appears to be reasonable to use cell lines with high PASKIN mRNA levels for further analysis *in vitro.*

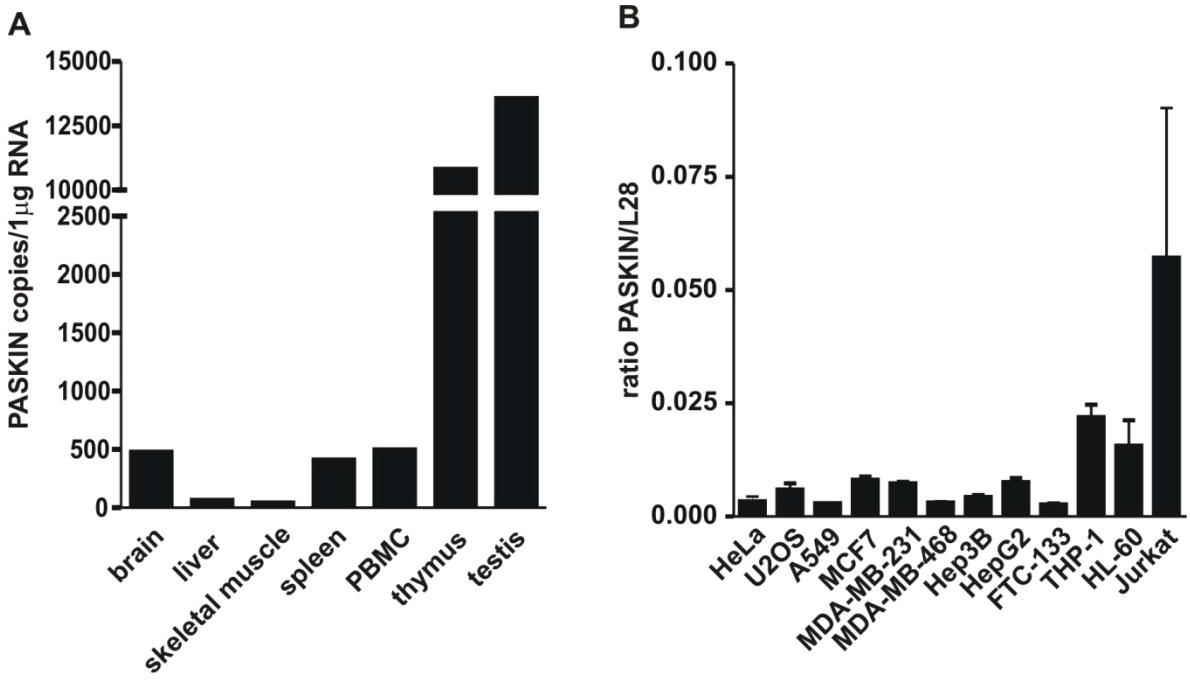

Fig. 1. PASKIN expression in various human tissues and cell lines. A.) RT-qPCR analysis of human tissue samples. Shown are number of PASKIN mRNA copies in cDNA obtained from 1 µg of total RNA. **B.)** RT-qPCR analysis of mRNA extracted from various human cell lines. Shown are mean values ±SEM of PASKIN over L28 mRNA copy numbers of 3 independent experiments.

PASKIN mRNA levels are independent of pro-inflammatory stimuli in cell lines.

The high expression levels of PASKIN in the human leukocyte cell lines THP-1, HL-60 and Jurkat mirror the situation in primary cells. This allows us to use these cells as *in vitro* models for investigating PASKIN regulation. To investigate putative PASKIN expression regulation upon activation of immune cells, we stimulated several mouse and human leukocyte cell lines with selected mitogens. Based on the previous experiment we chose the human monocytic leukemia line THP-1, the premyeloid line HL-60 and the T-cell leukemia line Jurkat (Gallagher et al. 1979; Gillis and Watson 1980; Tsuchiya et al. 1980). Additionally, we used the mouse pre-B-cell line 33.1.1 and the mouse B-lymphoid line K46 as putative candidates (Kim et al. 1979). The cell lines were stimulated for up to 72 hours with 1 µg/ml lipopolysaccharide (LPS) or 5 µg/ml phytohemagglutinin (PHA). Treated and untreated cells were collected 24 hours after seeding and after 24 hours, 48 hours and 72 hours time points. The isolated RNA was analysed by RT-qPCR for PASKIN and L28 expression levels. No changes in PASKIN expression by LPS or PHA stimulation were detected (Fig. 2A & B). Since prelimnary data showed minimal changes of PASKIN mRNA in HL-60 cells, the experimental procedure was optimized for LPS stimulation of HL-60 cells and the experiment was performed three times. TNF-α expression was measured to confirm successful

stimulation (Fig. 2C). Except for a down regulation of PASKIN expression after 72 hours in both stimulated and unstimulated cells, PASKIN levels were stable. However, the immortalized cell lines used might have lost the ability to react to stimulation by increasing PASKIN expression.

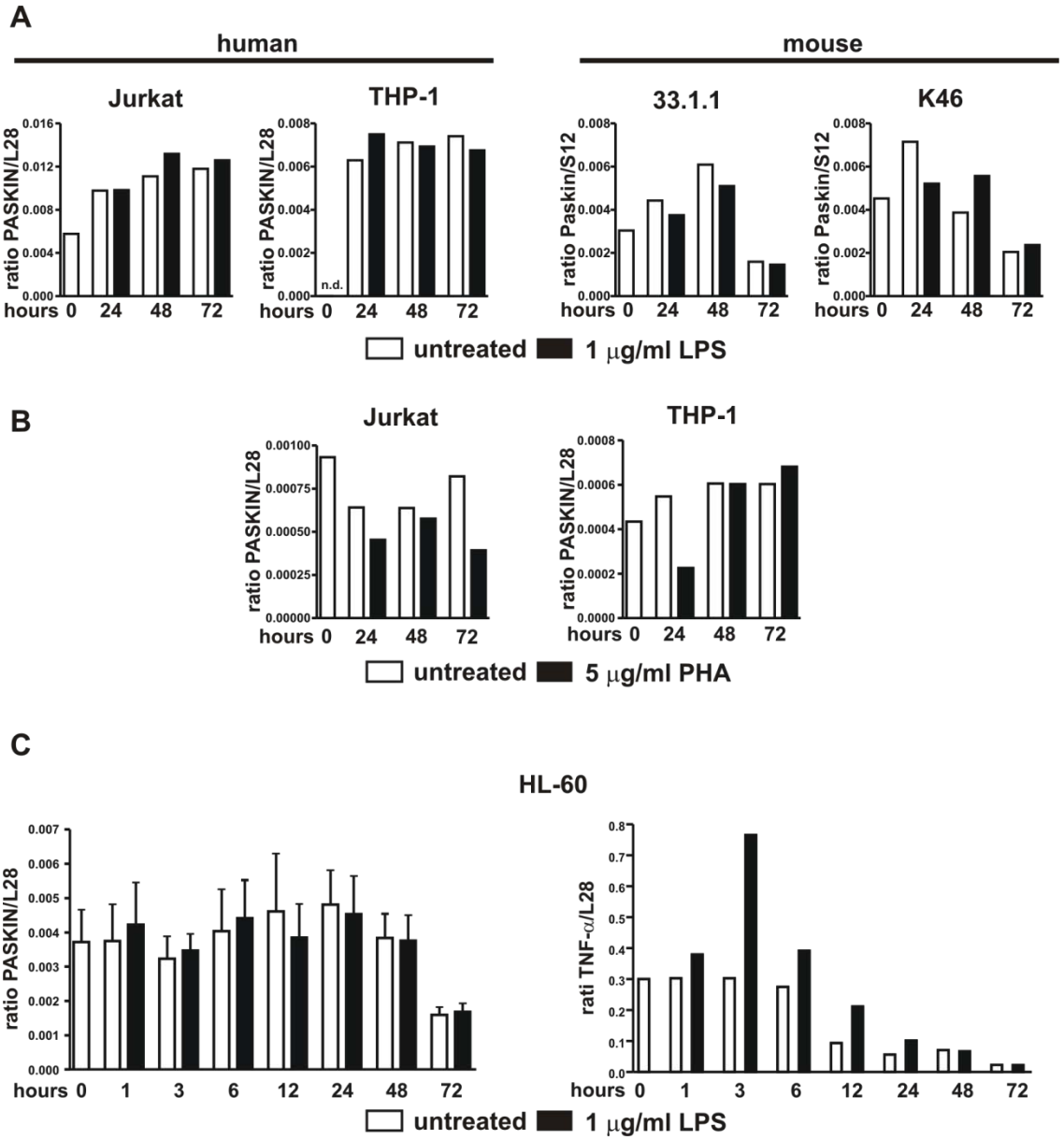

Fig. 2. Effects of immune cell line stimulation on PASKIN mRNA levels. A.) RT-qPCR analysis of cells stimulated with 1 µg/ml LPS for the indicated time. Shown are ratios of copy numbers of PASKIN mRNA levels compared to L28 or S12 mRNA, respectively. **B.)** The human cell lines Jurkat and THP-1 were stimulated with 5 µg/ml PHA for the indicated time. PASKIN mRNA copy numbers were determined by RT-qPCR and compared to L28 mRNA. **C.)** PASKIN and TNF-α mRNA levels compared to L28 mRNA in HL-60 cells stimulated with 1 µg/ml LPS for the indicated time. Shown are mean values ± SEM of 3 independent experiments. TNF-α was only determined in one experiment.

PASKIN is highly expressed in CD19 and CD3 positive cells but not in CD14 and CD16 positive cells

We found that leukocyte cell lines might not be an optimal *in vitro* model to investigate PASKIN expression regulation. Since PASKIN is expressed in a substantial amount in isolated human PBMCs we choose to use them for further experiments. However, PBMCs are a mixture of several cellular subsets. To identify the cellular subset expressing PASKIN we used magnetic beads to separate CD19, CD3, CD14 and CD16 positive cells from PBMCs isolated from buffy coats. Separation efficiency was confirmed by fluorescence-activated cell scanning (FACS).

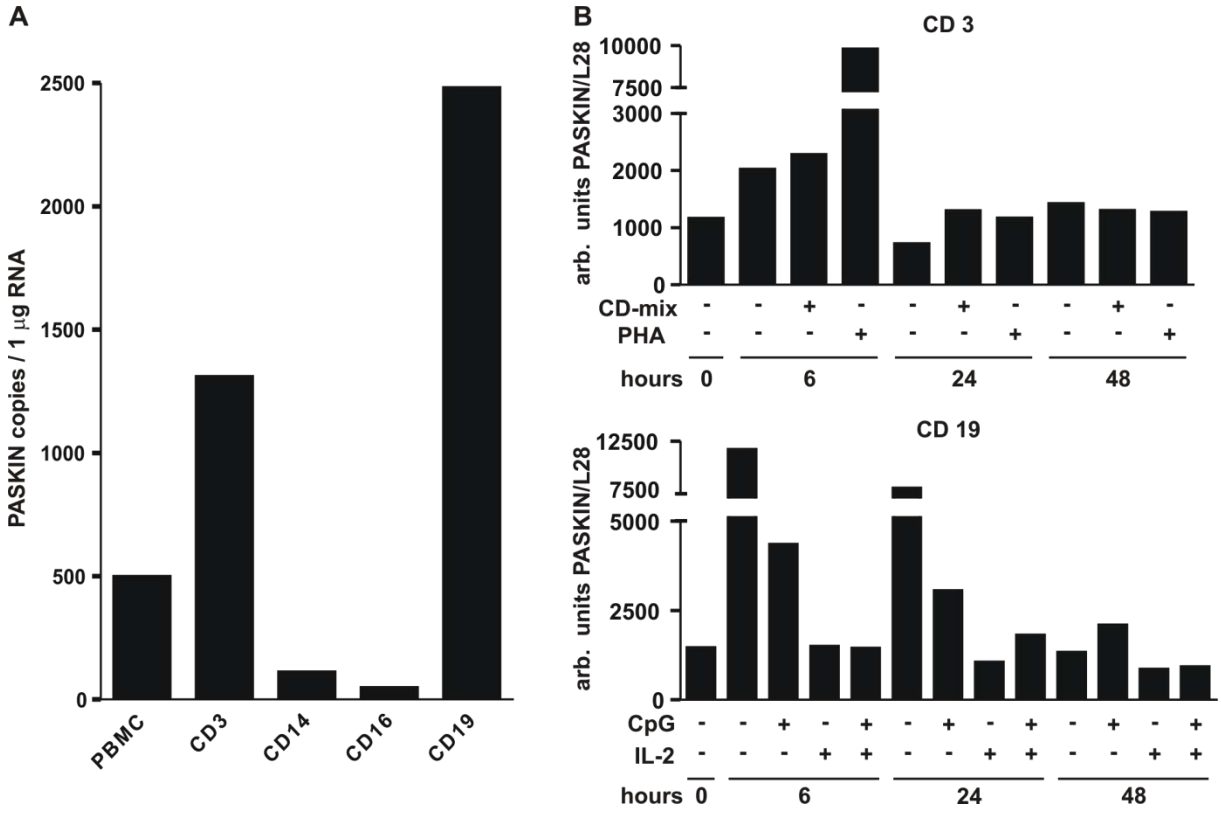

Fig. 3. Regulation of PASKIN mRNA levels in primary mononuclear blood cells. A.) PASKIN mRNA levels of PBMC and cell subsets separated by antibody-bound magnetic beads were analysed by RT-qPCR. Shown are number of PASKIN copies per 1 µg total RNA. **B.)** Separated CD3 and CD19 cells were stimulated with anti CD3/CD28/CD2 antibody mix (CD-mix) and PHA or unmethylated CpG oligonucleotides (CpG) and IL-2, respectively. Shown are RT-qPCR results of PASKIN mRNA levels compared to L28 mRNA levels in arbitrary (arb.) units defined as ($2^{-\Delta CT}$x100000).

CD3 positive cells were 98% pure consisting of 41% CD8 positive and 53% CD4 positive cells. For the negatively separated CD19 positive B-cells we achieved a purity of 99%. A

similar purity of 98% was determined for CD14 positive monocytes. The positive separation of CD16 positive NK-cells was only 60% pure. The impurity was mainly caused by monocytes and probably caused by the positive selection strategy. Nevertheless, RNA was isolated from all the separated cellular subsets. By qPCR analysis we found the highest PASKIN mRNA levels in CD19 positive cells followed by CD3 positive cells. CD14 and CD16 have mRNA levels of PASKIN, similar to organs like liver and spleen (Fig. 3A). However, it is possible that the results in NK-cells might be influenced by the presence of monocytes. Due to their high expression levels of PASKIN we used the separated CD3 and CD19 cells to investigate a putative cell type-specific regulation of PASKIN mRNA levels. The CD3 positive cells were stimulated with anti-CD3, anti-CD28, anti-CD2 antibody mix or PHA for 6 hours, 24 hours and 48 hours. The CD antibody mix allows costimulation of CD3, CD28 and CD2 that increases its potency. In a second experiment we stimulated primary CD19 cells with unmethylated CpG oligonucleotides (CpG), IL-2 or a combination thereof for the same time course. The selective stimulation of isolated primary CD3 positive and CD19 positive cells did not lead to an increase in PASKIN mRNA levels (Fig. 3B).

PASKIN mRNA is stable in whole PBMC cultures

The stimulation of isolated cell subsets might have no effect on PASKIN expression because important costimuli are missing in this setup. To investigate a putative role for PASKIN in activated lymphocytes in a more physiological setup than cell lines or separated cell populations we performed several experiments in a complete PBMC mixture. We isolated human peripheral blood mononuclear cells from buffy coats by Ficoll density gradient centrifugation. To identify an external stimulus for PASKIN expression in immune cells, the isolated PBMC mixture was stimulated with a mixture of antibodies against CD3, CD28 and CD2, CpG or PHA. The benefit of this variety of stimuli is the possibility to target different cellular subsets. Additionally, the complete mixture of PBMCs allows secondary cell-cell interaction effects putatively involved in PASKIN expression regulation (Fig. 4).

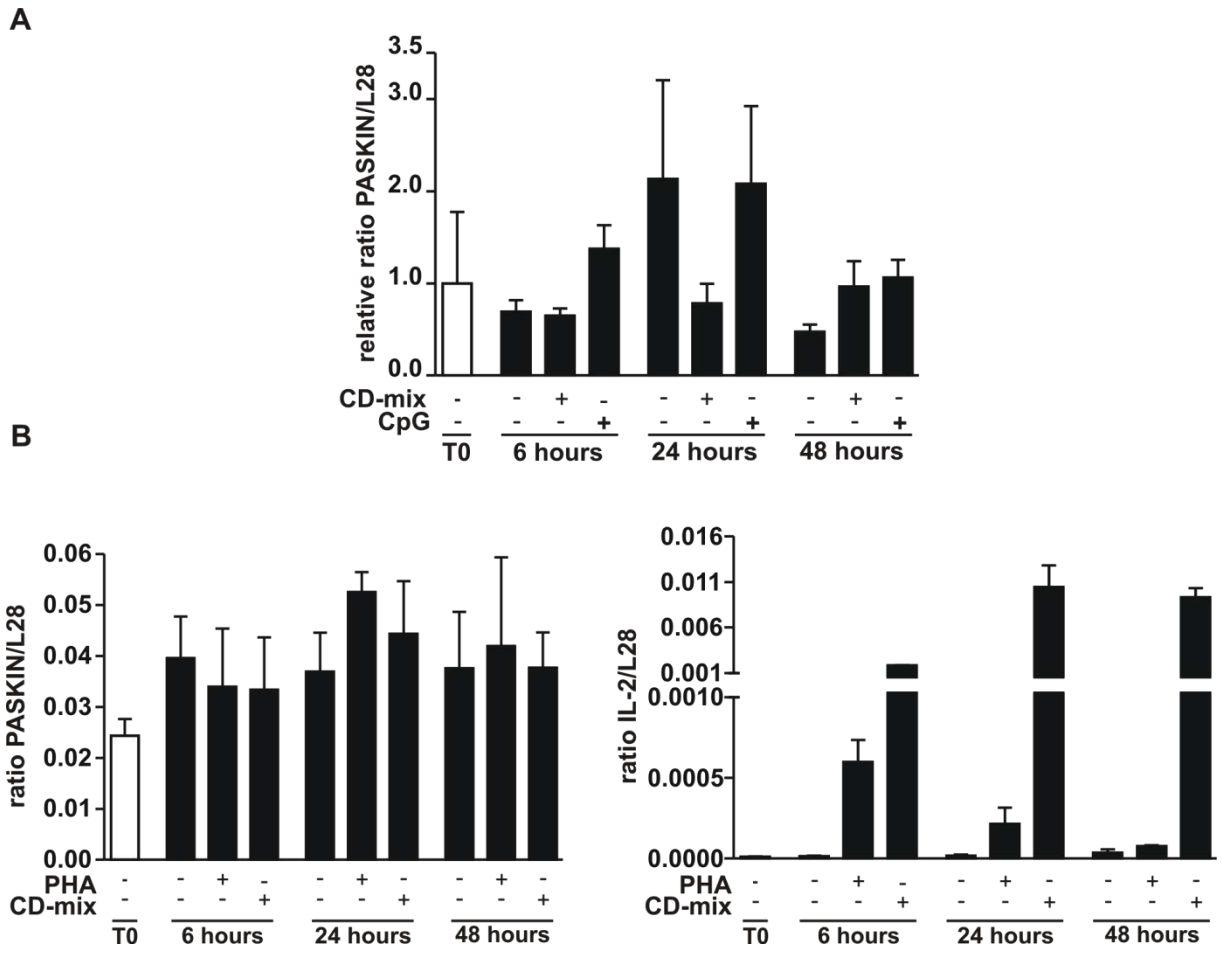

Fig. 4. Regulation of PASKIN mRNA levels in PBMC cultures. A.) Frozen PBMCs from 3 different donors were stimulated for the indicated time with CD-mix and unmethylated CpG oligonucleotides. "T0" indicates start of stimulation. Values at "T0" were set to 1. Shown are relative ratio of PASKIN mRNA levels compared to L28 mRNA. **B.)** Freshly isolated PBMC cultures were stimulated with PHA or CD-mix for the indicated time. "T0" indicates start of stimulation. Shown are ratios of PASKIN mRNA levels compared to L28 mRNA of 2 independent experiments with PBMCs from 2 donors.

Increased proliferation confirmed a successful stimulation. Additionally, we measured IL-2 mRNA levels as a positive control. Furthermore, we challenged whole PBMCs with the immunosuppressants dexamethason and cyclosporin A. We combined this treatment with PHA and LPS stimulation (Fig 5). Although the stimulated and inhibited PBMCs reacted as expected with increased or decreased proliferation activity, we could not find a change of PASKIN mRNA levels with any of these stimulations. This indicates that PASKIN, although expressed at high levels in CD3 and CD19 positive cells, is not regulated by T- or B-cells activation. However, a putative small change in PASKIN mRNA expression in one of the PBMC subsets could be masked by the abundance of the unaffected cell subsets.

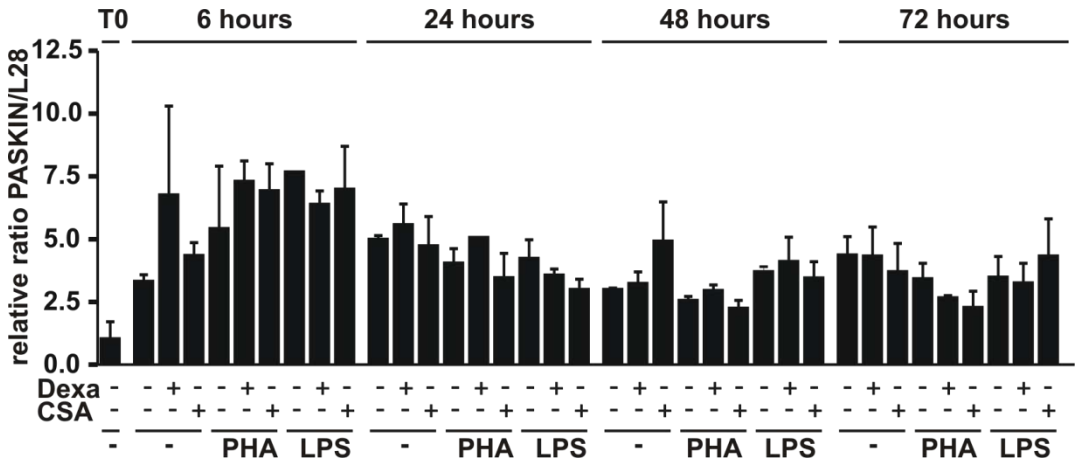

Fig. 5. Effects of immunosuppressants on PASKIN mRNA levels in PBMCs. PBMCs from 2 buffy coats were treated with dexamethason (Dexa) or cyclosporin A (CSA) and stimulated with PHA or LPS. "T0" indicates start of treatment. Shown are relative mRNA levels normalized to the values of untreated controls at "T0".

FACS experiments revealed PASKIN levels are independent of pro-inflammatory stimuli

To monitor PASKIN protein expression in the various subsets of isolated PBMCs, we developed an 8 color FACS staining strategy. We isolated PBMCs from two buffy coats and stimulated them with CD-mix, PHA or CpG over 72 hours. We collected samples right after isolation and after 24 hours, 48 hours and 72 hours. The samples were stained with differently labelled antibodies against the surface markers CD16/CD56, CD8, CD14, CD4, CD3 and CD19 and a labelled monoclonal antibody for the intracellular protein PASKIN. The eighth channel was reserved for the viability dye eFluor 450. The combination of these antibodies allowed us to gate for living populations of NK-cells (CD16), cytotoxic (CD8) and helper (CD4) T-cells (CD3), monocytes (CD14) and B-cells (CD19) and detect a putative change of PASKIN expressing cell populations upon stimulation. Analysis of PASKIN expression in the five cell populations mentioned above showed no regulation when treated with CD-mix, PHA or CpG (data not shown). This strongly suggests that PASKIN is not regulated in T-cells when stimulated with CD-mix or PHA, and B-cells do not respond to CpG treatment with a change of PASKIN expression. Furthermore, the FACS data suggest that inter-cell-population signaling of stimulated subpopulations is not inducing changes in PASKIN protein expression.

Response to LPS is altered in *Paskin* knockout mice

The high PASKIN levels in human PBMCs suggests a role for PASKIN in CD19, CD8 and CD4 positive cells T-cells. However, PASKIN expression is not regulated by stimulation of these cells. To address the question if PASKIN is necessary for an appropriate immune response, the reaction of *Paskin* knockout mice to immunization with DNP-KLH (2,4-dinitrophenyl hapten-keyhole limpet hemocyanin) or TNP-LPS (2,4,6-trinitrophenyl hapten-lipopolysaccharide) was analysed by the Group of Katapodis. In response to the immunization, some increase in IgM, but not in IgG could be observed after 6 to 8 days. In addition to the immunization experiments, cells isolated from spleens of *Paskin* knockout mice were stimulated with LPS. Interestingly, proliferative response of B-cells to LPS was increased in *Paskin* knockout mice. Additionally, T-cells of *Paskin* knockout mice showed a tendency to produce more IL-10 (Katapodis, personal communication).

Discussion

In mouse, considerable levels of Paskin mRNA have been found in testis, bone marrow, brain and thymus (Katschinski et al. 2003; Borter et al. 2007). The bone marrow and thymus play an important role in the development and maturation of leukocytes. Activation of lymphocytes induces changes in carbohydrate metabolism in these cells and induces proliferation (Mingari et al. 1984; Wang et al. 2011). The evidence from yeast showing PASKIN to be involved in changes of carbohydrate utilization and translation in response to an external stimulus in addition to the high basal mRNA levels in organs of the immune system led to our hypothesis that PASKIN might have a role in leukocyte activation (Rutter et al. 2002; Grose et al. 2007). We could show that human PASKIN mRNA levels similar to its mouse ortholog are high in testis, thymus, brain and spleen. The conservation of the tissue-specific regulation of PASKIN mRNA further supports a functional role for the tissue specific PASKIN expression. Furthermore, we found PASKIN mRNA levels to be high in peripheral blood mononuclear cells, and in the leukemic cell lines THP-1, HL-60 and Jurkat. This strongly indicates that the high expression of PASKIN detected in thymus, bone marrow and spleen is due to leukocytes which have high levels of PASKIN mRNA. Among the PBMC subsets, T- and B-cells showed substantially more PASKIN mRNA levels than monocytes and NK-cells. The results from the stimulation experiments suggest that PASKIN mRNA levels are not regulated during activation of lymphocytes. Additionally, we found no changes

in PASKIN expression levels in stimulated whole PBMC cultures. The high stable levels of PASKIN in lymphocytes might indicate that PASKIN is involved in a process preceding the activation or the activation itself. This is for example known for CD3, CD4 and CD8 (Teague et al. 1999). This would mean also that PASKIN is the metabolic switch mentioned above (Wang et al. 2011). With FACS experiments we were able to exclude that any, otherwise not detectable, shifts of PASKIN protein expression from one to another cell subset takes place upon stimulation with a variety of pro-inflammatory stimuli. Experiments in mice suggest some PASKIN-dependent differences in B-cell activity (Katapodis, personal communication). However, the relevance of these results remains unclear. The fact that till now no phenotype for *Paskin* knockout mice was found might be due to the high hygienic standards in modern animal husbandry. It has been shown before that mice with immune deficiency show no obvious phenotype under standard husbandry conditions (Liebscher et al. 2011). The almost excluded exposure to pathogens under these conditions might make a loss of Paskin obsolete. Challenging *Paskin* knockout mice with immunogens might reveal aberrations in the immune response caused by Paskin loss. The fact that PASKIN is expressed at high levels in cells that are specialized to react on an external stimulus with increased proliferation might be important. It has been proposed that the PAS-domain of PASKIN is inhibiting the kinase domain until binding of a yet unknown ligand (Amezcua et al. 2002). PASKIN expression in lymphocytes on the detected levels might be just enough and no additional increase of PASKIN mRNA and protein is needed. In this scenario the steady state levels of PASKIN protein are activated upon lymphocyte activation to fulfill their function. The identification of this stimulus would provide further insight into the function of PASKIN. To confirm or exclude a role of PASKIN in the immune response it is important to investigate the effect of PASKIN knockdown or over expression on the activation of lymphocytes. Interestingly, we found that the protein phosphatase *PPP1R7* gene, localized in the genome only 1 kilobase upstream of *PASKIN*, has a similar tissue expression pattern as PASKIN (Hofer et al. 2001; Katschinski et al. 2003). Additionally, we found cells expressing high levels of PASKIN also express higher levels of PPP1R7 (manuscript in preparation, chapter 5). This might indicate a putative co-regulation.

The *Drosophila* ortholog of PPP1R7, SDS22, has been shown to be important for cell polarity. Disrupted cell polarity goes in line with increased ERM family protein phosphorylation (Grusche et al. 2009). The ERM family members are also involved at the immunological synapse (Faure et al. 2004). One could imagine that PPPP1R7 and PASKIN might have a functional connection where they counteract each other at the immunological

synapse. High initial PASKIN expression levels that are not influenced by stimulation would make sense when PASKIN is involved in a process as spontaneous as rearrangement of the cytoskeleton in leukocytes. We further investigated a putative co-regulation of PASKIN and PPP1R7 with focus on the expression regulation and the evolutionary and functional characteristics of their bidirectional promoter (manuscript in preparation, chapter 5).

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5 MANUSCRIPT II (IN PREPARATION FOR SUBMISSON)

Evolutionary conservation of the PASKIN sequence and genomic architecture and the consequences on PASKIN and PPP1R7 expression

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Abstract

The human PAS kinase PASKIN has been identified by a BLAST search using the sequence of the bacterial O_2 sensor FixL. It has been shown that in yeast PASKIN has two orthologs involved in carbohydrate metabolism. In an *in silico* investigation we found PASKIN conserved from Excavata to human. This suggests that PASKIN is evolutionary more ancient than presumed till now. In a continuative study we found that the bidirectional promoter of *PASKIN* and *PPP1R7* appeared the first time in a common ancestor of sarcopterygian fish and tetrapodes, suggesting a more recent event. The mRNA expression analysis showed a strong coregulation of both genes regarding high and low expressing cell lines. We further studied the bidirectional promoter in a series of luciferase-based reporter assays in more detail. We found that the *PASKIN-PPP1R7* promoter had orientation-dependent activity. Furthermore, we were able to identify the genomic region partially responsible for high expression of PASKIN in immune cells observed before.

Introduction

Human PASKIN was identified based on a BLAST search with the sequence of the PAS-domain histidine kinase FixL of *Bradyrhizobium japonicum* (Hofer et al. 2001). Additionally,

an ortholog has been identified in *Drosophila*. Interestingly, two PASKIN homologs have been discovered in yeast (Rutter et al. 2001). While PASKIN is ubiquitously expressed, tissues of the immune system and testis show the highest expression levels (Borter et al. 2007). Little is known about the regulation of PASKIN mRNA expression. Da Silva Xavier and coworkers suggested that PASKIN mRNA expression might be regulated by glucose in insulin-secreting MIN6 cells or isolated rat islets (da Silva Xavier et al. 2004). However, *in vivo* experiments using *Paskin* wild-type and knockout mice did not show any Paskin mRNA regulation by high glucose and revealed that insulin expression is independent of Paskin (Borter et al. 2007). The human *PASKIN* gene contains 18 exons over 45 kb on chromosome 2. The first exon is localized 6 kb before the translational start site. The putative promoter region of *PASKIN* lying upstream of the first exon seems to be shared with the protein phosphatase 1 regulatory subunit 7 (PPP1R7) also called SDS22, a constellation that is conserved between mice and humans (Ceulemans et al. 1999). The close proximity of PPP1R7 and *PASKIN* genes might indicate a putative co-regulation (Katschinski et al. 2003). Such gene constellations are quite common in mammals (Adachi and Lieber 2002). Genes with 1 kb or less distance and a head to head orientation have been observed in more than 10% of the 23752 human genes investigated in an extensive study (Trinklein et al. 2004). Interestingly, it has been shown that the frequency of bidirectional promoters increases with the complexity of an organism (Koyanagi et al. 2005). Here we explored the taxonomical distribution of PASKIN among eukaryotes. Additionally, we show the evolutionary changes of the genomic organisation of the *PASKIN* promoter and its effect on the tissue specificity of PASKIN mRNA expression.

Materials and Methods

Identification of PASKIN orthologs

Several approaches were used to identify PASKIN orthologs. In one, we used known PASKIN sequences as queries in BLASTP and TBLASTN (Altschul et al. 1990; Altschul et al. 1997; Schäffer et al. 2001) searches with pair wise alignment of the NCBI non-redundant protein sequence and transcriptome databases (www.ncbi.nlm.nih.gov/BLAST/). In another approach, we employed the SUPERFAMILY genome assignments, based on a library of hidden Markov models (Gough et al. 2001), using a domain combination of an N-terminal PAS domain and a C-terminal protein kinase–like domain (http://supfam.mrc-lmb.cam.ac.uk).

Additional PASKIN orthologs were extracted using the MULTIZ whole-genome multiple alignment algorithm (Blanchette et al. 2004) implemented in the UCSC Genome Browser (Rhead et al. 2010) and using homology data from the Ensembl Compara database (Vilella et al. 2009). All potential PASKIN orthologs were validated using Conserved Domain Database searches for the presence of a PAS domain and serine/threonine kinase domain (Marchler-Bauer et al. 2009).

Cell culture

HeLa, U2OS, A549, MCF7, MDA-MB-231, MDA-MB-468, Hep3B, HepG2 and FTC-133 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma). HL-60, Jurkat and THP-1 were cultured in RPMI-1640 (Sigma) at 37°C. In all cases, medium was supplemented with 10% FCS (Invitrogen), 50 IU/ml penicillin and 50 µg/ml streptomycin (Sigma).

RNA extraction from cell lines

Cells (1×10^6) were seeded in 10 cm dishes and grown under the growth conditions described above for 48 hours. Cells were lysed in 2 ml solution D (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol). Total RNA was extracted as described before (Chomczynski and Sacchi 1987). mRNA was reverse transcribed with AffinityScript multiple temperature reverse transcriptase (Agilent). cDNA levels corresponding to 2.5% of the reverse transcribed RNA were analysed by Reverse Transcriptase quantitative PCR (RT-qPCR) using a SybrGreen qPCR reagent kit (Sigma) and a MX3000P light cycler (Stratagene). The initial concentration of templates was determined using standard curves of serially diluted standards.

Design of promoter-reporter luciferase vectors

The human *PASKIN* promoter was amplified by PCR with Phusion high fidelity DNA polymerase (Finnzymes) using the following primers:

hPASKProFw 5'-AATTCCATGGTGGCTGCTCTTCCC-3'

hPASKProRv 5'-TATACCATGGAGCCCTTCCGCGCTTTTATC-3'.

The PCR product was digested with NcoI and subsequently purified by gel electrophoresis using a gel extraction kit (Qiagen). The PCR product was ligated with T4 ligase (Fermentas) for 1 hour into the NcoI site of the pGL3Basic vector (Promega). Transformation of XL1-Blue MRF' electrocompetent bacteria was perfomed by electroporation with GenePulserII

(BioRad). Correct integration was verified by sequencing (Microsynth). Deletion products were generated by digestion with PstI, SmaI, SacI, NheI and subsequent religation. Plasmids were purified with the Nucleobond kit (Macherey-Nagel).

Transient transfection and reporter gene assays

Electroporation was performed with a GenePulserII (BioRad). Cells in exponential phase were electroporated with 50 µg reporter construct DNA and 800 ng of pRLSV40 Renilla luciferase reporter vector (Promega). Electroporation conditions were 1000 µF, 250 V, with no parallel resistance. Subsequently, cells were resuspended in culture medium. Polyethyleneimine (Polysciences) transfection was performed as described previously (Stiehl et al. 2006). Cells were harvested in passive lysis buffer (Promega) and luciferase reporter gene activity was determined using the dual-luciferase reporter system according to the manufacturer's instructions (Promega).

Results

PASKIN has a broad taxonomic distribution

In yeast two PASKIN paralogs exist whereas in *Drosophila,* mouse and human only one PASKIN ortholog is conserved (Rutter et al. 2002). In yeast and mammals, PASKIN function has been investigated (Schläfli et al. 2009). However, in the widely used model organism *Drosophila melanogaster* only little research on Paskin has been done (Shaukat et al. 2012). Another model organism that has been proven useful in elucidating regulatory mechanisms for another PAS protein, HIF-1, is *Caenorhabditis elegans*. This nematode would provide a powerful tool to further investigate PASKIN functions. However, we could not find any PASKIN ortholog in the free-living nematode *C. elegans*, additional *Caenorhabditis* species as well as in parasitic nematodes, including *Loa loa, Brugia malayi* and *Ascaris suum.* Intrigued by this finding, we investigated other completely and nearly completely sequenced genomes from a series of different taxonomical classes for the presence of PASKIN (Table 1). To identify previously unannotated PASKIN orthologs, we performed BLAST searches employing established and newly confirmed full-length PASKIN sequences. In contrast to the serine/threonine kinase domain, PAS domains display a poor sequence conservation. Consequently, the BLAST searches often resulted in identifications of other serine/threonine kinases. Domain identification and functional annotation were done using the Conserved Domain Database to confirm the presence of a predicted PAS domain N-terminal to the serine/threonine kinase (Marchler-Bauer et al. 2009). We considered candidate genes containing only a PAS domain lying upstream in close proximity of a gene containing only serine/threonine kinase domain as false annotations and we claim that these two genes are in fact a long reading frame for PASKIN. We found this situation in *Nematostella vectensis* and *Branchiostoma floridae*. In the coelacanth *Latimeria chalumnae,* no full-length PASKIN sequence is annotated but the *Latimeria Paskin* ortholog resides on JH127163:473,322-701,824. However, the position is very close to the edge of the contig JH127163, as a consequence of the incomplete *L. chalumnae* genome sequence, rendering prediction of the full-length *Paskin* gene difficult. We found PASKIN to be conserved in all investigated fungi and metazoa. Peculiar exceptions are nematodes in which we could not identify a PAS domain-containing kinase. Until now, we have no explanation for the absence of Paskin in nematodes, but the lack of an otherwise highly conserved protein in the nematode animal lineage is not entirely unprecedented (Hoogewijs et al. 2012).

Taxon	Representative species	Identified PASKIN in GeneBank
Mammalia		
	Homo sapiens	NP_001239048.1
Sauropsida		
	Anolis carolinensis	GAGA01509546.1
	Gallus gallus	XP_422656.3
Amphibia		
	Xenopus tropicalis	XP_004917848
Osteichthyes		
	Latimeria Chalumnae	proposed: JH127163:473,322-701,824
	Danio rerio	XP_003197983.1
	Takifugu rubripes	XP_003975651.1
Urochordata		
	Ciona intestinalis	XP_002131885.1
Cephalochordata		
	Branchiostoma floridae	XP_002593656.1 PAS is not annotated
Hemichordata		
	Saccoglossus kowalevskii	XP_002731565
Echinodermata		
	Strongylocentrotus purpuratus	XP_791921.2
Arthropoda		
	Drosophila melanogaster	NP_611864.1
Nematoda		
	Loa loa, Brugia malayi,	
	Ascaris suum	not found
	Caenorhabditis sp.	not found
Placozoa		
	Trichoplax adhaerens	XM_002114479.1
Cnidaria		
	Nematostella vectensis	XP_001622647.1 (PAS); XP_001622648.1 (Kinase)
	Hydra magnipapillata	XP_002170763.2
Choanoflagellida		
	Monosiga brevicolli	XP_001746536.1
Fungi		
	Saccharomyces cerevisiae	NP_009385.2 / NP_014597.1
Protozoa		
	Amoebidium parasiticum	GAKF01053888.1
Excavata		
	Trypanosoma congolense	CCC92697.1

Table 1. Proteins identified as PASKIN in various eukaryotic classes.

It is important to point out that we also identified Paskin in *Trypanosoma congolense*, a unicellular parasite causing trypanosomiasis in animals. Trypanosomes belong to the protozoan group excavate (Cavalier-Smith 2009). Therefore, PASKIN might have been already present in a common ancestor of plants and animals, suggesting that PASKIN conservation is evolutionary more ancient than previously anticipated. However, in this study we did not focus on PASKIN conservation in plants.

PASKIN expression is ubiquitous but shows a high tissue-specific variation

The widespread taxonomic distribution of PASKIN suggests a strongly conserved function. A role of PASKIN in a fundamental cellular process is supported by the ubiquitous expression in human tissue (Hofer et al. 2001). However, PASKIN mRNA levels show a high tissue-specific variation in mice (Katschinski et al. 2003; Borter et al. 2007). This indicates that certain tissues like sperm cells have a higher demand for PASKIN, although it is used ubiquitously. The tissue specificity might therefore give some indications about the function of PASKIN. To investigate the evolutionary conservation of PASKIN expression we measured PASKIN mRNA levels by RT-qPCR in human tissue samples. As in the mouse, we found the highest levels of Paskin mRNA in testis and thymus. We observed less PASKIN mRNA in spleen and brain. In skeletal muscle and liver, substantially less PASKIN expression was detected. The abundance of PASKIN mRNA in isolated peripheral blood mononuclear cells (PBMCs) was similar to spleen and brain. Interestingly, we found high PASKIN mRNA levels in CD19 and CD3 positive cells (Fig. 1. & chapter 4). Importantly, the strong expression in testis and thymus was confirmed in an additional species, *Gallus gallus*, by EST profiles extracted from the UniGene database (data not shown). Little is known about the regulation of *PASKIN* gene expression and no appropriate cell line model has been found so far, except for mouse MIN6 cells in which PASKIN expression has been reported to be induced by glucose in some (da Silva Xavier et al. 2004) but not other studies (Borter et al. 2007). Therefore, we screened a series of human cell lines for PASKIN mRNA expression levels. These expression analyses demonstrated that leukemic cell lines have a higher PASKIN expression than other cell lines (Fig. 1A), confirming the results we obtained from human tissue and PBMCs.

It has been suggested before that *PASKIN* might share a common bicistronic promoter with protein phosphatase 1 regulatory subunit 7 (*PPP1R7*) which is located only 1 kilobase upstream of *PASKIN* on the opposite strand (Hofer et al. 2001). Although, PPP1R7 mRNA levels are generally higher they are detected in the same tissues as Paskin (Katschinski et al.

2003). The *PPP1R7-PASKIN* promoter has the typical characteristics of a bidirectional promoter like lying in a CpG island and transcriptional start sites located on opposite strands separated by 1kb (Fig. 1A). Since this might be also of functional importance we determined PPP1R7 mRNA levels and confirmed that PASKIN and PPP1R7 have a very similar mRNA expression pattern in the investigated cell lines (Fig. 1B). The putative co-regulation in human tissues and cell lines prompted us to further investigate the *PASKIN-PPP1R7* promoter region.

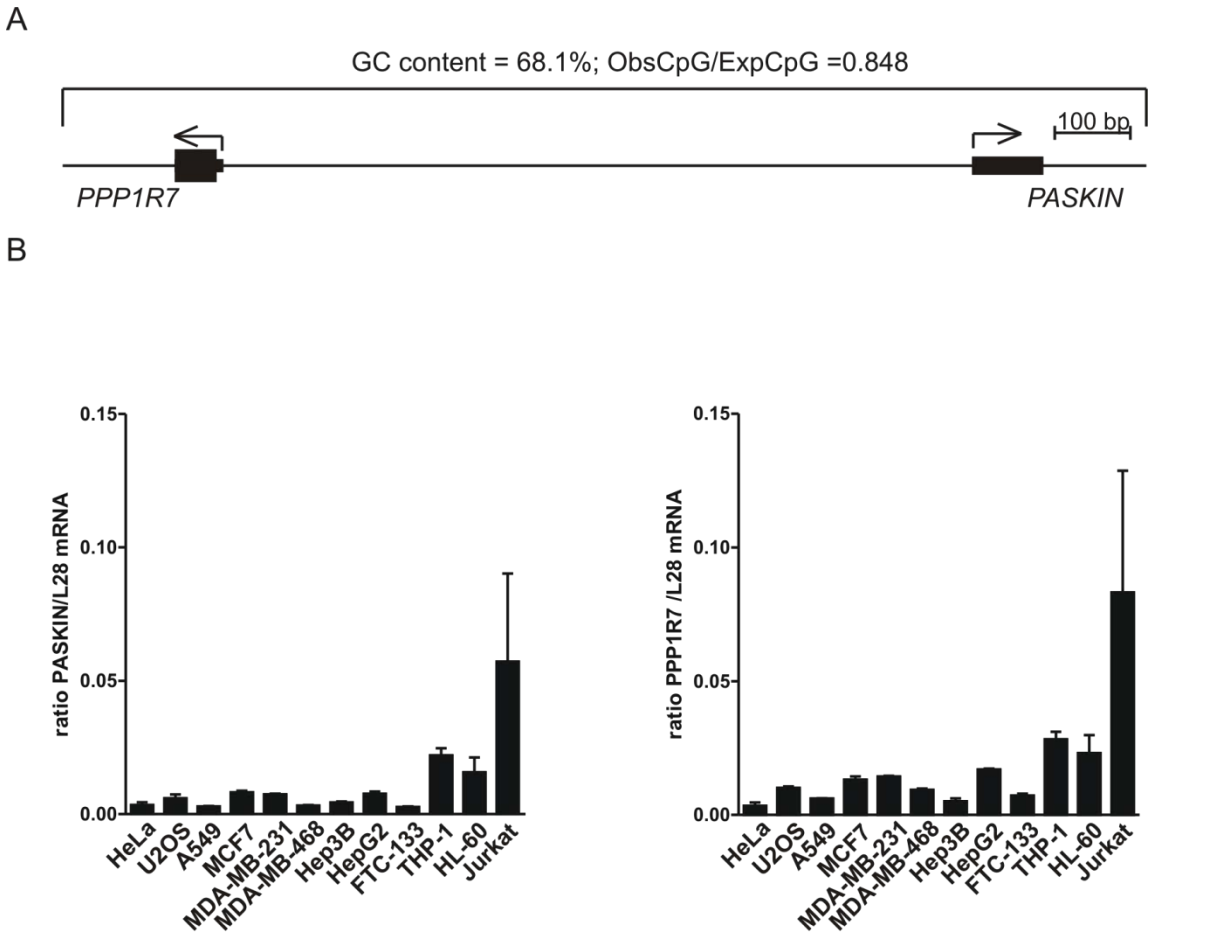


Fig. 1. The genomic arrangement of *PASKIN* and *PPP1R7* might promote co-regulation. A.): Shown is the genomic locus of *PASKIN* and *PPP1R7*. Arrows indicate the transcriptional start sites of each gene. GC content of the indicated region and ratio of observed CpG over expected CpG content is shown. Untranslated and translated sequences are indicated by thin and broad bars. **B.)** PASKIN mRNA levels were determined by RT-qPCR and compared to the mRNA levels of the ribosomal protein L28. Shown are ratios of 3 independent experiments ± SEM.

The genomic constellation of *PASKIN* and *PPP1R7* is conserved in tetrapoda

The common expression pattern of PASKIN and PPP1R7 in human cell lines might indicate a co-regulation of these two genes. It has been shown that the chromosomal localisation of *PASKIN* and *PPP1R7* is conserved between mouse and human (Ceulemans et al. 1999). In an

attempt to learn more about the peculiar genomic constellation of *PASKIN* and *PPP1R7*, we assessed synteny conservation using the Ensembl Compara tool of all newly identified PASKIN homologs. Unannotated genes upstream of *PASKIN* were compared with the non-redundant protein database using BLAST. We considered the constellation as conserved when i no other gene was predicted between *PPP1R7* and *PASKIN* and when ii both genes were located on opposite strands facing different directions. Although we found some variability in the distance between the two genes in mammals, the relative position of the two genes was conserved. Synteny analyses further showed that the constellation was also conserved in other tetrapods. In fish, we obtained a more heterogeneous picture. Although *PPP1R7* and *PASKIN* were located on the same chromosome in Actinopterygi they were separated by more than 25 Mb. Intriguingly, in *Latimeria chalumnae* the constellation of *PASKIN* and *PPP1R7* was conserved (Table 2). No synteny conservation could be observed in more basal taxa.

Superclass	Class	Representative species	Constellation
Tetrapoda			
	Mammalia	*Homo sapiens*	conserved
		Mus musculus	conserved
	Sauropsida	*Gallus gallus*	conserved
		Anolis carolinensis	conserved
	Amphibia	*Xenopus tropicalis*	conserved
Osteichthyes			
	Sarcopterygii	*Latimeria chalumnae*	conserved
	Actinopterygii	*Danio rerio*	not conserved
Agnatha			
		Petromyzon marinus	not conserved

Table 2. Conservation of the genomic constellation of *PASKIN* and *PPP1R7*. Constellation was considered to be conserved when the two genes are located on opposite strands with no other genes annotated in between.

The activity of the *PASKIN-PPP1R7* promoter region is orientation-dependent

In silico analysis using the ENCODE data (Thomas et al. 2007; Rosenbloom et al. 2013) from the UCSC Genome Browser (*hg19*) (Kent et al. 2002) demonstrated that the potential *PASKIN-PPP1R7* promoter region contains high DNaseI hypersensitivity, promoter-associated histone modification marks and a strong transcription factor occupancy pattern, all of them indicating substantial promoter activity. To further explore the putative bidirectional promoter of *PASKIN* and *PPP1R7* we cloned a 1038 bp sequence reaching from the start codon in the first exon of *PPP1R71* to the first exon of *PASKIN* into a luciferase reporter vector. We designed one construct containing the 1038 bp insert in *PASKIN* orientation (construct I) and one containing the same sequence inverted in the *PPP1R* orientation (construct VI). Additionally, we made two deletion constructs by PstI (construct II) or NheI (construct III) digestion followed by religation. This resulted in two constructs in *PASKIN* orientation missing a central part of 395 bp or 321 bp, respectively. To study the region more proximal to the transcriptional start site we generated two short promoter constructs of 261 bp (construct IV) and 204 bp length (construct V) (Fig. 2A). HeLa and U2OS cells were transfected with the described luciferase reporter constructs. We observed that luciferase activity driven by the whole 1038 bp orientated in *PASKIN* direction (construct I) was only slightly different from luciferase activity in the cells transfected with an empty control in both cell lines (Fig. 2B). Intriguingly, we found a very high activity when luciferase expression was driven by the same DNA sequence orientated in *PPP1R7* direction (construct VI). This is in line with the endogenous expression patterns where PPP1R7 mRNA levels are usually higher than PASKIN mRNA levels (Katschinski et al. 2003). However, the differences observed in the activity of reporter constructs are more pronounced, suggesting the presence of site-directed elements in the investigated sequence that lead to different expression levels of PPP1R7 and PASKIN. The deletion caused by PstI digestion (construct II) resulted in an increase of luciferase activity compared to construct I, potentially indicating a role of the distal *PASKIN* promoter elements, responsible for the high PPP1R7 expression, which are located closer to the transcriptional start site in this construct. Deletion of a DNA fragment of a similar size, but more proximal to *PASKIN*, resulted in increased luciferase activity compared to the full-length promoter, albeit to a lesser extent than the one caused by the PstI deletion. This could indicate the presence of a putative inhibitory element in the region that was deleted in construct II but is still present in the deletion of construct III.

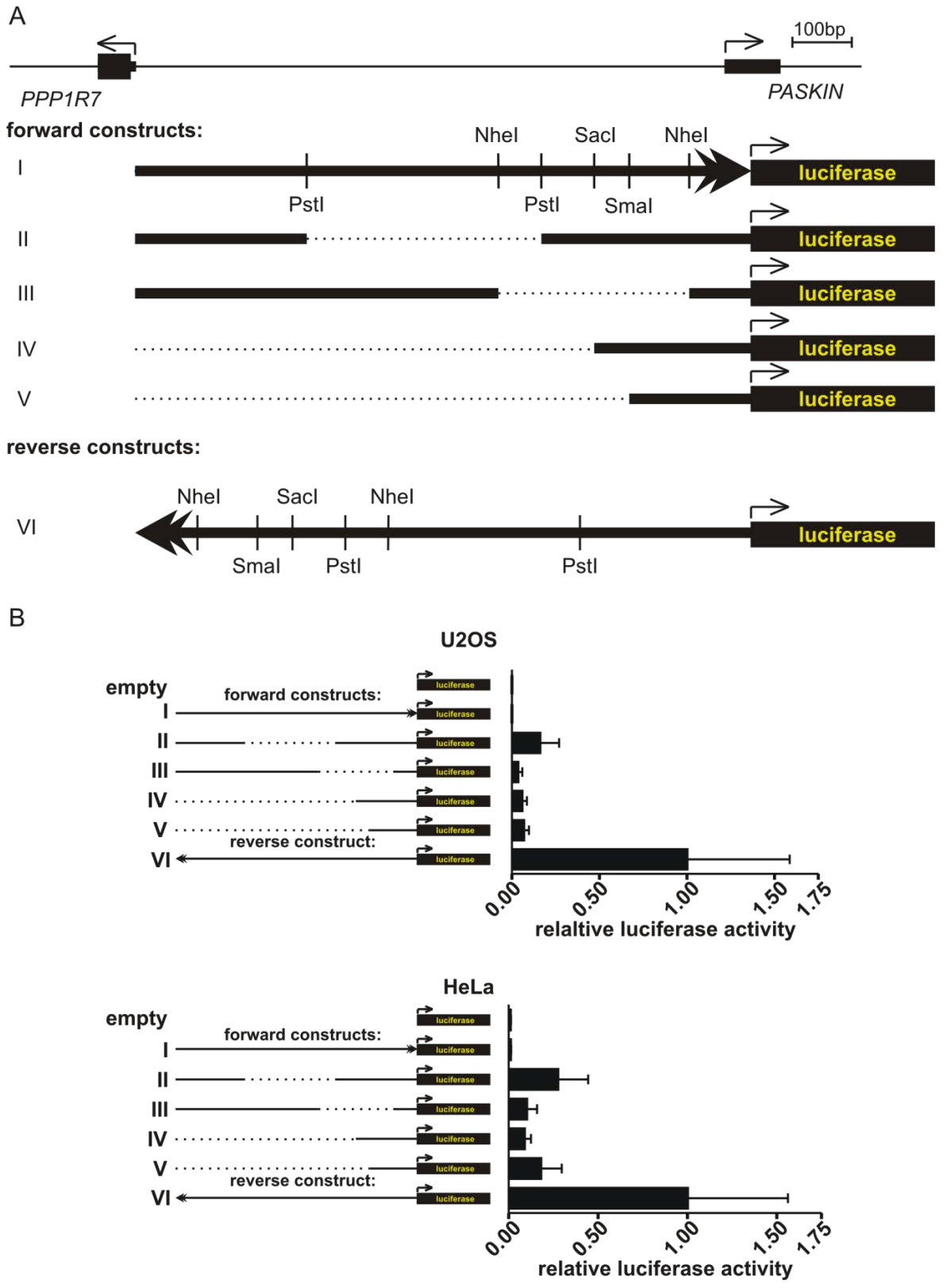


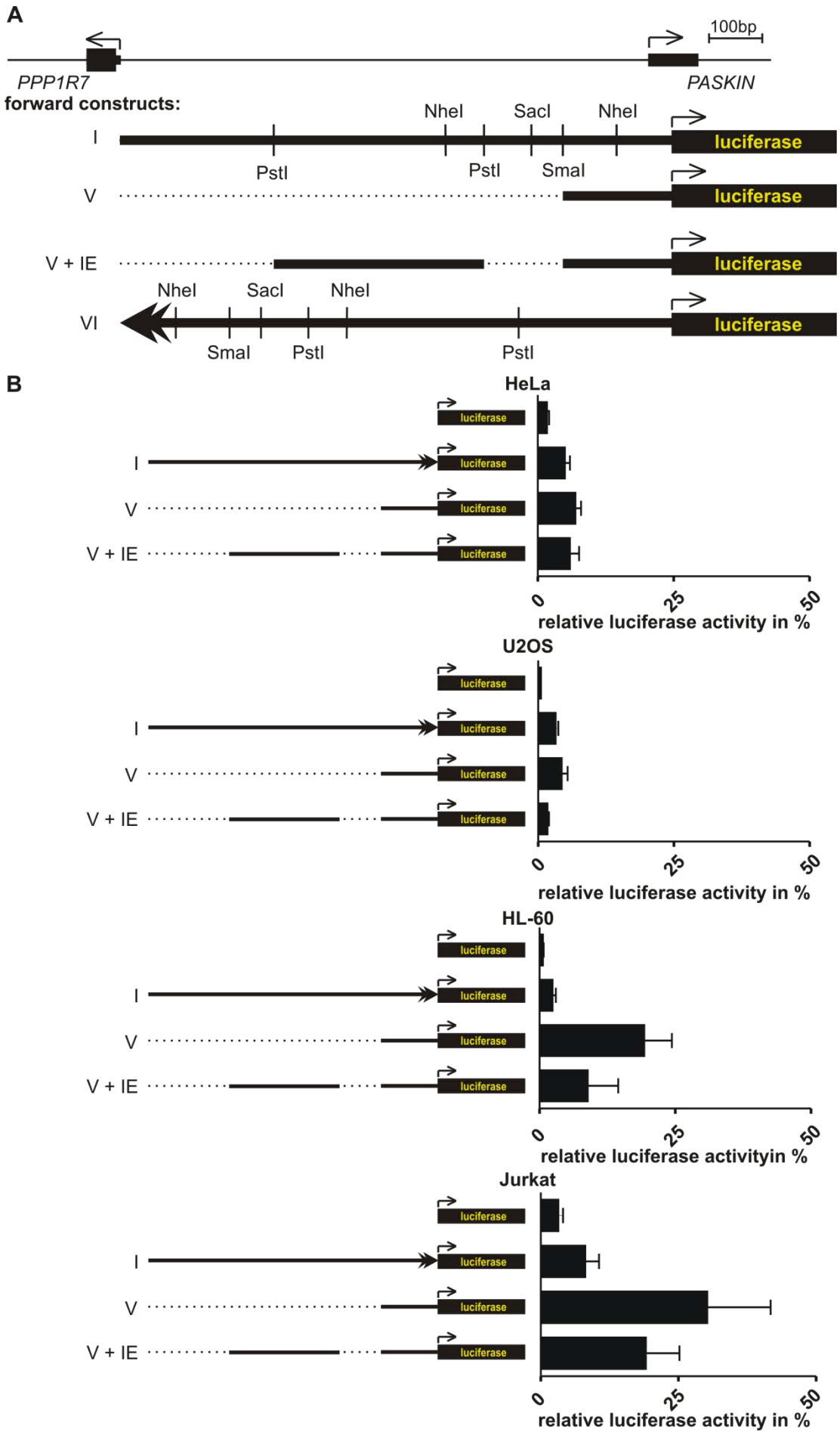
Fig. 2. Design and activity of the *PASKIN* promoter reporter constructs. A.) Transcriptional start sites relative to the *PASKIN-PPP1R7* genomic loci are indicated by arrows. Sequences used in the indicated. Reporter constructs are arranged according to their genomic position. Dashed lines indicate missing sequences in the respective reporter constructs. Relative positions of the restriction sites used in the design of the various constructs are marked. **B.)** Shown are the reporter constructs described in A and their relative activity compared to the construct VI of 3 independent experiments. Error bars show ± SEM.

Additional reporter gene assays illustrated that the short sequences from the *PASKIN* proximal region of 261 and 204 bp displayed an activity lower than the Pst1 deletion, but higher than the full promoter and the NheI deletion. Of the two proximal promoter constructs, the shorter 204 bp sequence showed the highest activity and was therefore studied in more detail.

The minimal promoter shows differential activity in selected cell lines

The short 204 bp promoter fragment of construct V showed a robust but weak activity compared to the full-length promoter in PPP1R7 orientation. However, PASKIN mRNA levels are lower in HeLa and U2OS than in the leukemic cell lines HL-60 and Jurkat. To further investigate the role of the 204 bp construct in cell line specific expression of PASKIN, we compared U2OS, HeLa, HL-60 and Jurkat cells transiently transfected with empty vector and construct V. As a putative inhibitory element could contribute to the tissue specific regulation of PASKIN expression, we designed an additional construct consisting of the piece deleted in construct II (IE) and the 204 bp short promoter (construct V + IE) (Fig. 3A). Moreover, we transfected the construct VI. Whereas luciferase driven by the full-length 1053 bp promoter is similar in all four cell lines, *PASKIN* minimal promoter activity in relation to construct VI was higher in HL-60 and Jurkat compared to U2OS and HeLa. Additionally, we found that inclusion of the putative inhibitory element results in a reduction of luciferase activity. The strength of this effect was variable in the four cell lines but no striking difference between leukocytes and other cell lines could be observed. Collectively, the reporter gene assays indicate that the proximal 204 bp stretch of construct V is mainly responsible for the tissue-specific variations of PASKIN mRNA levels and the putative inhibitory element has only a minor effect on cell line-specific activity.

Fig. 3. *PASKIN-PPP1R7* promoter reporter constructs in various cell lines. A.) Relative position to the genomic locus of *PASKIN* and *PPP1R7* and design of the reporter constructs used in B are shown. **B.)** The relative activity of the reporter constructs in four different cell lines is shown in % of the activity of construct VI. Shown are the values of 3 independent experiments ± SEM.


A
100bp
PPP1R7
PASKIN
forward constructs:
NheI
SacI
NheI
I
luciferase
PstI
PstI
SmaI
V
luciferase
V + IE
luciferase
NheI
SacI
NheI
VI
luciferase
SmaI
PstI
PstI
B
HeLa
luciferase
I
luciferase
V
luciferase
V + IE
luciferase
0
25
50
relative luciferase activity in %
U2OS
luciferase
I
luciferase
V
luciferase
V + IE
luciferase
0
25
50
relative luciferase activity in %
HL-60
luciferase
I
luciferase
V
luciferase
V + IE
luciferase
0
25
50
relative luciferase activityin %
Jurkat
luciferase
I
luciferase
V
luciferase
V + IE
luciferase
0
25
50
relative luciferase activity in %

Discussion

The functional role of mammalian PASKIN is not well understood and the proposed mechanisms of PASKIN regulation are controversial (da Silva Xavier et al. 2004; Borter et al. 2007). We were interested in the *PASKIN* promoter region and its influence on PASKIN expression. Our comprehensive *in silico* analyses revealed that PASKIN is strongly conserved in animals, fungi and even protozoa as well as choanoflagellates. Although plant species were excluded from our analysis, it is worthwhile to note that PAS serine/threonine kinases are far more abundant in plants. In *Arabidopsis* 6 PAS serine/threonine kinases have been predicted (Ichimura et al. 2002). Therefore, PAS serine/threonine kinases are existing in a great variety of species. This suggests that PASKIN might have a role in basal cellular processes. This is further supported by the ubiquitous expression of PASKIN, suggesting a general function in various cell types which might be adapted in some tissues for specific purposes like regulation of insulin in pancreatic β-cells. PASKIN has been proposed as an energy sensor because it has been observed that PASKIN mRNA and protein levels as well as activity are increased by high glucose concentration (da Silva Xavier et al. 2004; Fontés et al. 2009; da Silva Xavier et al. 2011). However, we found no glucose-dependent change in Paskin expression in MIN6 cells or isolated pancreatic islets (Borter et al. 2007). Additionally, Paskin mRNA levels are reduced in certain regions of the hypothalamus and the neuroblastoma cell line N2A (Hurtado-Carneiro et al. 2013). This suggests that the glucose dependent regulation of the expression and probably the enzymatic activity of PASKIN is highly context dependent. No mechanism has been proposed by which glucose affects PASKIN expression and activity. Additionally, the various species with PASKIN have different strategies of regulating energy metabolism. So, it remains unclear whether the occasionally observed effect of glucose on PASKIN expression is of any relevance or the observed changes in PASKIN expression are secondary, caused by more general effects of glucose on certain cells. In line with this hypothesis, phospholipids rather than glucose showed regulation of PASKIN kinase activity (Schläfli et al. 2011). In summary, no reliable regulator of *PASKIN* gene expression has been identified thus far.

Here, we found that the high tissue specificity of Paskin expression in mouse is conserved in human (Borter et al. 2007) suggesting a physiological relevance. We found that in cell lines PPP1R7 has a similar expression pattern as PASKIN, supporting the previously proposed co-regulation of PASKIN and PPP1R7 (Hofer et al. 2001; Katschinski et al. 2003). A controlled co-regulation would suggest a functional relationship between PASKIN and PPP1R7. We

showed that the constellation of the two genes was conserved in tetrapods. Among fish, we found *PASKIN* and *PPP1R7* in the same constellation only in the Sarcopterygi *Latimeria chalumnae*. In Actinopterygii, *PASKIN* and *PPP1R7* were located on the same chromosome and 28 Mb apart. Since *Latimeria chalumnae* shares a common ancestor with tetrapoda, this suggests that in the common ancestor of *Latimeria* and the extant tetrapods a rearrangement of the *PASKIN* containing chromosome resulted in this peculiar positioning of *PASKIN* and *PPP1R7*. This event took place after the radiation of Actinopterygii and Sarcopterygii. Although *Latimeria* is more closely related to tetrapods than other fishes, the closest water-living relative of tetrapods is the lungfish *Protopterus annectens* (Amemiya et al. 2013), whose genome sequence is not available yet. The terrestrialization of tetrapods was associated with major physiological changes and the potential co-regulated PASKIN and PPP1R7 expression due to the newly arranged chromosomal proximity of both genes might have been beneficial for tetrapods. However, a more ancient functional connection between PASKIN and PPP1R7 cannot be excluded.

The yeast ortholog of PPP1R7 is SDS22 that acts as a regulatory subunit of the ortholog of protein phosphatase 1 in yeast, Glc7. Glc7 is involved in glycogen accumulation and cell wall integrity (Feng et al. 1991; Andrews and Stark 2000), very similar to the functional context of the yeast PASKIN orthologs Psk1 and Psk2. However, the role of SDS22 seems to be to direct Glc7 to other process mainly involved in mitosis and chromosome stability (Ohkura and Yanagida 1991; Peggie et al. 2002). Recently, it has been shown that a loss of PASKIN promotes DNA damage when the spindle assembly checkpoint is weakened (Shaukat et al. 2012). It is therefore not unlikely that PASKIN and PPP1R7 are not only genetically but also functionally connected. The kinase activity of PASKIN and the regulation of phosphatase activity by PPP1R7 would provide a relatively simple mechanism for functional connection. We investigated the putative co-regulation in more detail with a series of luciferase reporter constructs. The sequences we analysed correspond to the promoter region of *PASKIN* and *PPP1R7*, excluding other more distal regulatory elements for example in the introns of the two genes. Furthermore, luciferase reporter assays are insensitive to chromatin organisation-dependent effects. We found that the luciferase expression driven by the promoter sequence in *PASKIN* orientation is very low, representing the low endogenous PASKIN mRNA levels. In contrast, the endogenous expression of PPP1R7 is usually higher than PASKIN expression. This was nicely reflected in the much higher luciferase expression driven by the promoter in *PPP1R7* orientation. The region proximal to the transcriptional start site of *PPP1R7* might contain directional regulatory elements responsible for the orientation-dependent differences.

This is supported by the effect of the 395 bp deletion equally distant from the *PASKIN* and the *PPP1R7* transcriptional start site. This deletion seems to enable these active elements responsible for the high expression in *PPP1R7* direction and also to promote luciferase activity in *PASKIN* orientation. A potential explanation might be that the deletion brings these elements closer to the transcriptional start site or the deletion causes the loss of an actively inhibiting element acting only in *PASKIN* direction. A third possibility would be an element blocking the *PPP1R7* elements to act on the *PASKIN* part of the promoter. However, a deletion of a similar sized DNA stretch of 321 bp closer to the *PASKIN* transcriptional start site shows less activity than the more central deletion, suggesting that the position of the deletion is more important than the proximity of the putative more active elements in the *PPP1R7* proximal region. The lower activity of the two constructs only consisting of 261 bp and 204 bp, respectively, close to the transcriptional start site of *PASKIN* further supports the importance of elements in the proximal *PPP1R7* promoter. In the leukemic cell lines HL-60 and Jurkat we found a similar activity for the full-length promoter in *PASKIN* direction like in the U2OS and HeLa cell lines. However, the shortened 261 bp promoter showed a higher activity, relative to the activity of the full promoter in *PPP1R7* orientation, in the two leukemic cell lines than in HeLa and U2OS. The addition of the 261 bp fragment, containing a putative inhibiting element seems to have a similar inhibitory effect in all cell lines, indicating that this element indeed has an inhibitory function but, at least in the luciferase constructs, does not exhibit different cell type-specific activity. These two observations strongly suggest the presence of elements in the 261 bp close to the *PASKIN* transcriptional start site that are more active in the two leukemic cell lines and substantiate that this region is essential for tissue specific *PASKIN* expression. However, the endogenous inhibitory element might be deactivated in lymphatic cells by epigenetic mechanisms that would not be reflected in our reporter gene assays.

This is the first study reporting the taxonomical distribution and promoter regulation of PASKIN. The conserved bidirectional promoter of *PPP1R7* and *PASKIN* might suggest a closer functional relation between the two genes than presumed till now.

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6 MANUSCRIPT III: (UNPUBLISHED)

Weakening of the spindle checkpoint by Mad2 depletion in mammalian *Paskin* knockout cells

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Abstract

Mammalian PASKIN has a unique protein architecture since it combines a PAS domain with a serine/threonine kinase domain. It has been proposed that PASKIN is involved in glucose metabolism and in regulation of protein synthesis. In *Drosophila*, loss of Paskin increases p53 dependent apoptosis induced by DNA damage in a chromosome instability background caused by depletion of the spindle checkpoint protein Mad2. A connection between PASKIN and the spindle checkpoint has not been investigated in the mammalian system before. Herein, we found higher levels of Mad2 in *Paskin* knockout MEF cells than in wildtype MEFs. We could not detect any differences in viability, proliferation or DNA damage response in *Paskin* knockout or wildtype cells depleted of Mad2. Although we cannot exclude a functional link between Paskin and Mad2, these results suggest that the depletion of Mad2 and Paskin is not sufficient in mammals to induce the effects observed in *Drosophila*.

Introduction

PAS domain containing proteins exhibiting a sensory function are widespread. The PAS domains are often combined with an effector domain and induce a function upon signal

receiving (Henry and Crosson 2011). The mammalian PASKIN is unique in vertebrates in combining a PAS domain with a kinase domain (Hofer et al. 2001). The serine/threonine kinase domain of PASKIN is putatively inhibited by its PAS domain (Rutter et al. 2001). A structural analysis of the PAS domain of PASKIN suggests the ability to bind small compounds (Amezcua et al. 2002). In the current model PASKIN binds a yet unknown ligand. This subsequently leads to the derepression of the kinase domain and subsequently to increased auto-and target phosphorylation (Schläfli et al. 2009). Several in vitro kinase targets involved in translational regulation and the regulation of insulin secretion have been identified and we could show that PASKIN is able to increase translation efficiency *in vitro* (An 2006; Eckhardt et al. 2007; Schläfli et al. 2011). However, it is still unknown what the function of PASKIN is *in vivo*. A role for PASKIN in glucose sensing in β and α cell function is controversial (da Silva Xavier et al. 2004; Borter et al. 2007; da Silva Xavier et al. 2011). Furthermore, mouse Paskin mRNA levels in pancreas are rather low compared to other organs like testis, thymus and bone marrow (Katschinski et al. 2003; Borter et al. 2007).

MAD2 is an important part of the spindle checkpoint. In a so called open state MAD2 is binding MAD1. Unattached kinetchores recruit MAD2-MAD1 complexes. The binding of the MAD2-MAD1 complex to the kinetochores induces the accumulation of more free MAD2 at the binding site (Shah et al. 2004). Free MAD2 sequestered by MAD1 is able to bind CDC20 and therefore induce the assembly of the mitotic checkpoint complex (MCC) (De Antoni et al. 2005). The MCC inhibits the anaphase promoting complex (Sudakin et al. 2001), blocking the progression of the cell cycle to anaphase (Pines 2011).

Recently, it was discovered that a double knockdown of Paskin and Mad2 leads to increased lethality in *Drosophila* (Shaukat et al. 2012). Flies with a Mad2 knockdown alone as well as with a Paskin knockdown alone are viable. Mad2 knockdown results in a chromosome instability (CIN) phenotype with increased occurrence of defective anaphase chromatide separation. The knockdown of Paskin resulted in no obvious phenotype (Shaukat et al. 2012). Also no phenotype was observed for *Paskin* knockout mice (Katschinski et al. 2003; Hao et al. 2007). In contrast, homozygous mammalian *Mad2* knockout mice die during early embryonic stages (Dobles et al. 2000). No research was reported so far on the effects of a double knockdown in mammalian cells. Further investigation in *Drosophila* revealed that double knockdown of Paskin and Mad2 in wing discs results in increased tissue loss due to apoptosis. It was observed that the double knockdown resulted in increased activation of the DNA damage repair machinery. A triple knockdown of Paskin, Mad2 and p53 suggests that

the increased apoptosis is p53 dependent. However, no mechanism was presented on how Paskin knockdown exhibits this effects in a Mad2 depleted background (Shaukat et al. 2012). In this study, we intended to elucidate the role of Paskin in a CIN background in the mammalian system.

Materials and Methods

Cell culture

Mouse embryonic fibroblast (MEF) cells generated from *Paskin* +/+ and *Paskin* -/- mice (Katschinski et al. 2003) at embryonic day 14 (E14) were cultivated in Dulbecco's modified Eagle's medium-high glucose, containing 4.5 g/l glucose (Sigma). The medium was supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 50 IU/ml penicillin, and 50 µg/ml streptomycin (Sigma).

Cell lysates and immunoblotting

Cells were washed once and harvested by scraping into ice cold PBS. Subsequently, cells were washed twice in ice cold PBS and whole cell lysates were generated by heating for 5 minutes in 1% SDS in PBS. Samples were centrifuged for 5 minutes at maximum speed to pellet cell debris. The concentration of the supernatant was determined by BCA protein assay (Pierce). SDS PAGE was used to separate proteins. Proteins were electro transferred to a PVDF membrane (Carl Roth). The following antibodies were used to detect the respective protein: mouse-anti-β-actin (Sigma), rabbit-anti-MAD2 (Bethyl). To detect the primary antibodies, secondary antibodies goat-anti-mouse or anti-rabbit coupled with horseradish peroxidase were used (Pierce). Supersignal West Dura substrate was used for chemiluminescence signal development (Pierce). Chemiluminescence was recorded with a LAS-4000 CCD camera (Fuji). Quantification was performed with Quantity One software (BioRad).

Indirect immunofluorescence

MEFs (5×10^4 cells) were seeded on coverslips in 12-well plates and let attach for 16 hours. Normal cell culture medium was exchanged with medium containing 0 µM, 2 µM, 4 µM or 8 µM etoposide. Cells were exposed to etoposide for 1 hour, then rinsed with PBS and fixed at

room temperature with 2% paraformaldehyde in PBS for 5 minutes. The fixed cells were permeabilized with 100% methanol at −30°C for 5 minutes. After blocking with 3% bovine serum albumin (BSA, Invitrogen) for 1 hour, the mouse primary antibody anti-γH2AX (JBW103, Upstate, Charlottesville, VA) in PBS with 3% BSA was added. The slides were incubated at room temperature for 1 hour and washed three times with PBS before secondary anti-mouse Alexa488 (Molecular Probes, Invitrogen) was added for 1 hour. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (Dapi, Sigma) for 30 minutes followed by three washing steps with PBS. Slides were mounted in 30 µl MOWIOL and dried over night in the dark. Epifluorescence was analyzed using an Axio fluorescence microscope (Zeiss) and images were captured with fixed exposure times. Pictures of each condition were taken and combined into stacks. The stacks were than analysed automatically for cell number and number of foci using imageJ software.

Short hairpin RNA constructs and lentiviral infections

HEK293T cells (5×10^6) were cultured in a 75 cm^2 culture flask for 24 hours before transfection. with pLKO.1, containing sh sequences against Mad2 (Sigma), pLP1, pLP2 and PVSV-G using polyethyleneimin (Polysciences) as described before (Stiehl et al. 2006). The following day, medium was replaced and lentiviral particles were collected by filtering the supernatant through a 0.45 µm filter. The viral stocks were stored at 4°C. Viral stock was mixed with an equal amount of fresh medium containing 12 µg/ml polybrene. After 24 hours, medium was replaced with fresh medium. After 72 hours selection was started with 1 µg/ml puromycin. The concentration of puromycin was later increased to 5 µg/ml.

MTT conversion assay

Cells ($5 \times 10^{3)}$ were seeded in a 96 well plate with 120 µl of medium. After 24 hours, medium was exchanged with medium containing 1 ng/ml, 10 ng/ml, 100 ng/ml and 1 µg/ml of nocodazole (Sigma). After 16 hours, 15 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution (5 mg/ml) was added. Plates were shaked for 5 minutes and subsequently incubated for 5 hours at 37°C in a cell culture incubator. Supernatant was removed and the remaining crystals were dissolved in 150 µl of isopropanol. The conversion of MTT was quantified in an ELISA reader.

Results

DNA damage response is independent of Paskin in primary mouse embryonic fibroblasts

Paskin knockout mice show no gross alteration in phenotype and normal fertility (Katschinski et al. 2003; Eckhardt et al. 2007). However, animals under standard husbandry conditions are exposed to little environmental stimuli. A loss of the ability to react to such stimuli may not show a phenotype under these circumstances.

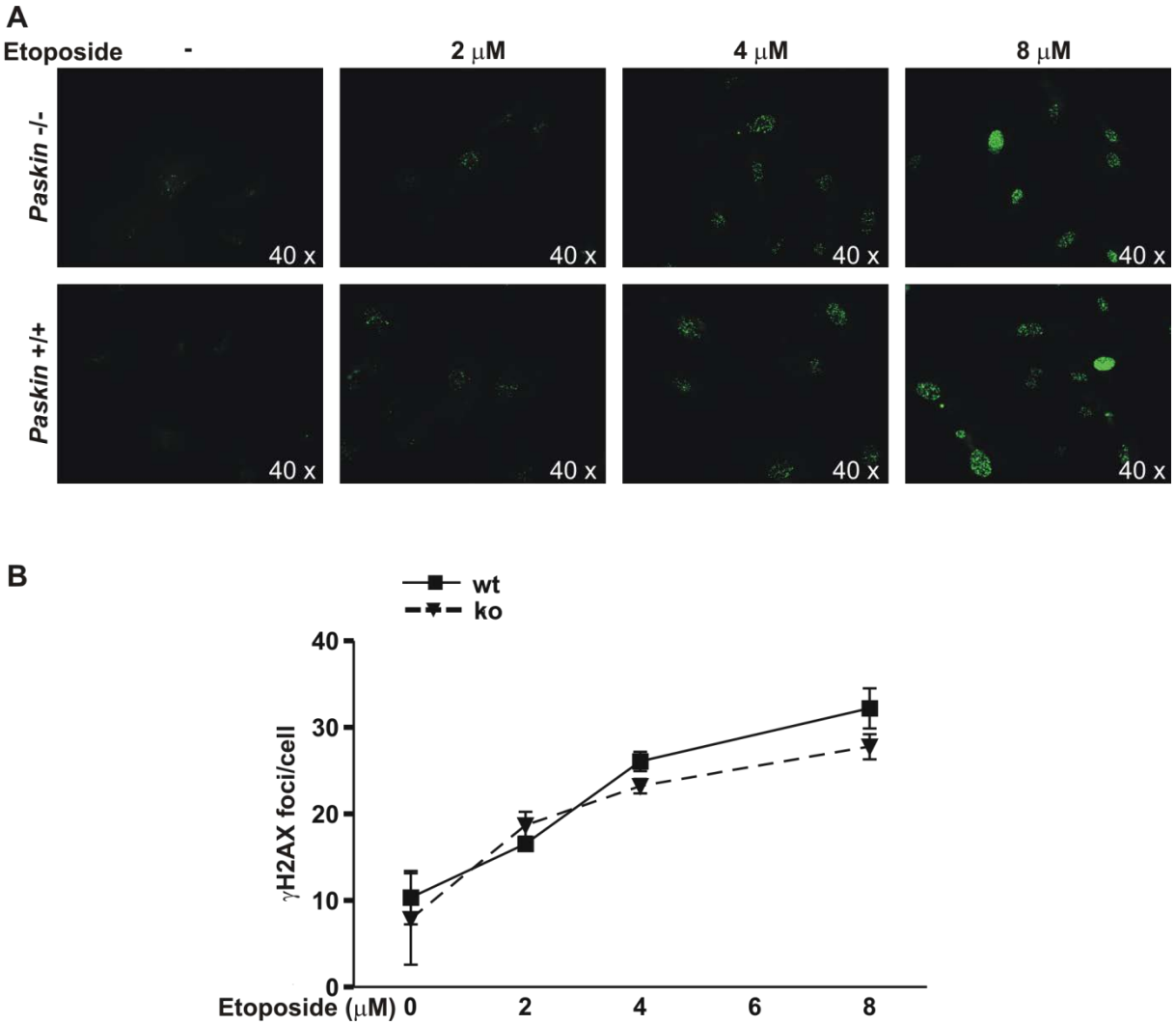

Fig. 1. Etoposide treatment of primary mouse embryonic fibroblasts (MEF) from *Paskin* knockout and wildtype mice. A.) Primary independent MEF cell lines derived from 2-3 *Paskin* knockout or wildtype animals were treated with 0, 2, 4 and 8 µM etoposide for 1 hour. Subsequently, cells were fixed and stained for γH2AX by immunofluoresence. Shown are representative pictures of γH2AX immunofluoresence. More γH2AX accumulates in foci with increased etoposide concentration. **B.)** Quantification of γH2AX foci numbers in etoposide treated primary MEFs from *Paskin* knockout mice and wildtype littermates. Shown are mean values ± SEM from independent MEF lines derived from 2 *Paskin* knockout mice and 3 wildtype littermates.

In a first attempt to clarify a putative role of Paskin in the spindle checkpoint we made use of primary mouse embryonic fibroblasts (MEFs) derived from *Paskin* knockout mice. To investigate if the knockout of *Paskin* in mice has an effect on DNA damage susceptibility, we exposed pools of isolated primary MEF cells derived from 2 *Paskin* knockout mice and from 3 wildtype littermates to 0, 2, 4 and 8 µM etoposide for 1 hour. The treatment was stopped by washing and immediate fixation with 2% paraformaldehyde. The fixed etoposide exposed cells were then stained for γH2AX by immunofluorescence. Etoposide has been shown to increase DNA single and double strand breaks by inhibiting topoisomerase II (Wozniak and Ross 1983; Hande 1998). Phosphorylation of the histone variant H2AX is a fast and early step in the response of the DNA damage machinery induced by double strand breaks (Sak and Stuschke 2010). We evaluated the damage response by determining the number of nuclear foci formation in the stained cells. As expected, number of γH2AX positive foci per cell were increasing with etoposide concentration. However, the number of foci per cell was independent of the genotype. These observations suggest that loss of Paskin does not increase DNA damage (Fig. 1).

Knockdown of Mad2 in immortalized *Paskin* knockout MEFs

Depletion of MAD2 in human cells leads to a fast loss of proliferation ability and eventually to a nearly complete loss of viability because cells fail to divide properly (Michel et al. 2004). *Mad2* knockout in mice results in early embryonic lethality (Dobles et al. 2000). Dobles and colleagues observed *in vitro* that the highly mitotic inner cell mass of the *Mad2* knockout embryos stops to proliferate after embryonic stage E6.5. Interestingly, trophoblast giant cells, which lose their mitotic ability at E4.5 survived longer (Dobles et al. 2000). We used lentiviral particles to deliver short hairpin (sh) constructs targeting Mad2 into immortalized mouse embryonic fibroblast derived from *Paskin* knockout mice or wildtype littermates. To obtain pool of clones with reduced Mad2 expression we had to increase puromycin concentrations gradually and extend time of selection (Fig. 2 A&B). The difficulty in obtaining Mad2 depleted cells might be due to negative selection caused by the previously reported growth disadvantage of Mad2 depletion in mammalian cells. It is worth to mention that the screening for Mad2 reduction in the infected cells revealed that MEF cells derived from *Paskin* knockout animals show a higher Mad2 protein expression compared to MEF cells derived from wildtype litter mates (Fig. 2C).

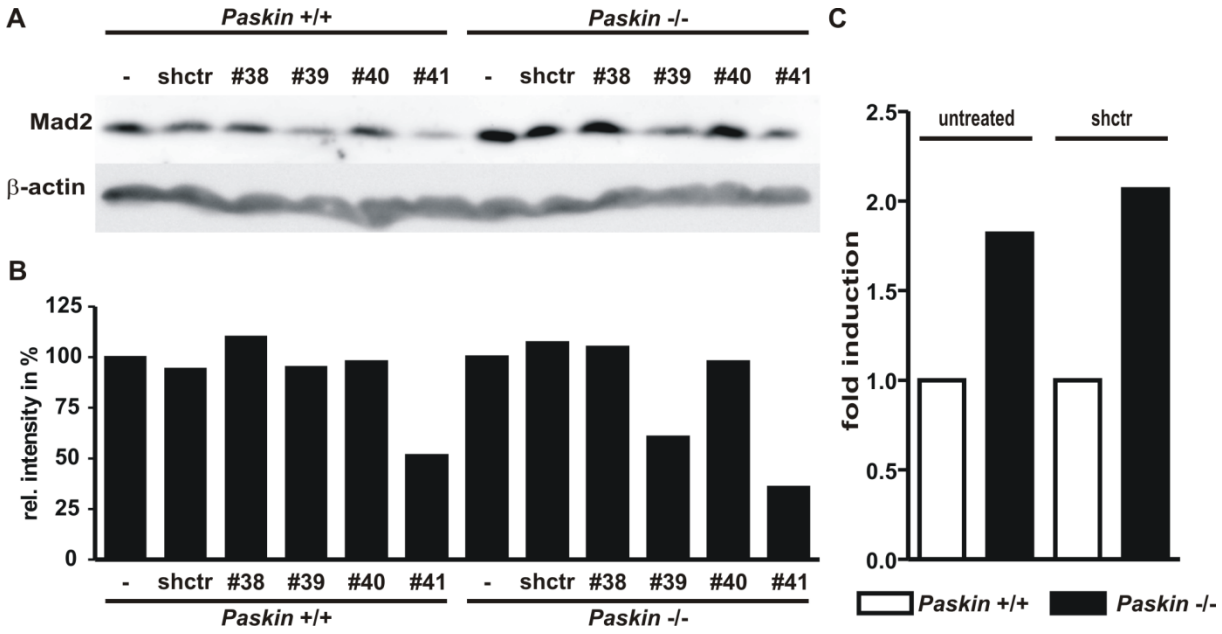

Fig. 2. Analysis of shMad2 infected immortalized *Paskin* knockout and wildtype MEF cells. A.) Immunoblot analysis of Mad2 and β-actin in untransduced cells (-) and cells transduced with lentiviral particles containing shctr or one of 4 different shMad2 constructs numbered #38, #39, #40 and #41. **B.)** Quantification of western blot shown in (A). Mad2 expression is shown relative to β-actin levels. Values for untransduced cells have been set to 100% for each *Paskin* genotype. **C.)** Mad2 relative expression levels in untreated and shctr MEFS from wildtype animals and from *Paskin* knockout mice. Shown are fold induction normalized to the respective wildtype control.

No alterations of Mad2 mRNA levels were observed in *Paskin* knockout cells (data not shown). This suggests a Paskin dependent regulation of Mad2 on the translational or protein stability level. However, the cells in which this was observed are all derived from the same pool of immortalized MEFs from one donor. Therefore, these experiments should be repeated independently to confirm the effect of Paskin on Mad2. Additionally, the effect of Paskin knockdown and overexpression in other cell lines should be determined.

Impact of Mad2 depletion in a *Paskin* knockout background on nocodazole response

MEFs with a reduced Mad2 expression mentioned above showed no growth phenotype in standard cell culture conditions. However, it has been reported previously that Mad2 depleted cells show an alteration in their response to nocodazole. Mad2 depleted cells fail to undergo mitotic arrest upon microtubule depolymerisation induced by nocodazole (Meraldi et al. 2004). The depolymerisation of microtubules blocks the formation of the metaphase spindle and triggers the spindle assembly checkpoint.

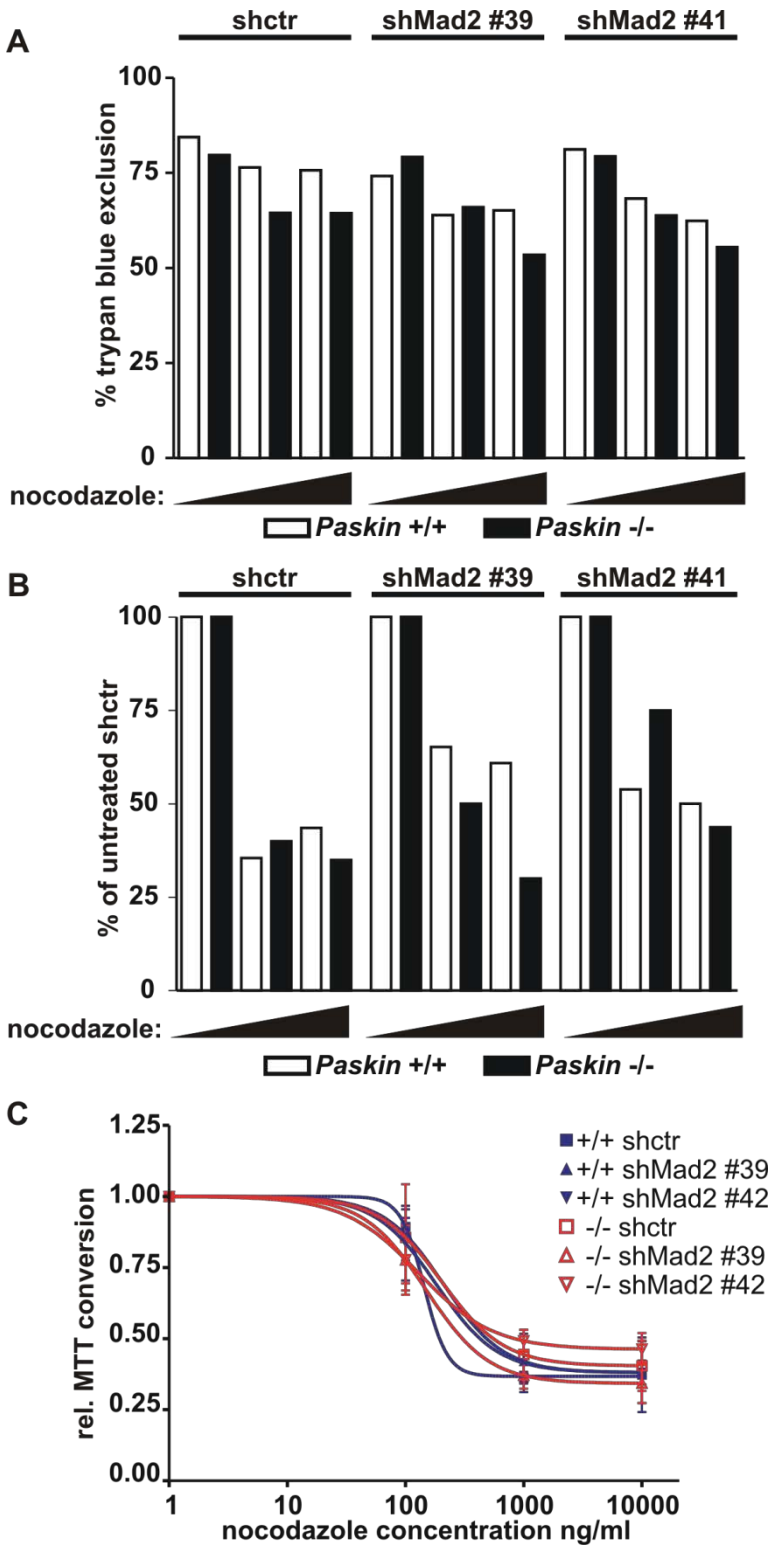

Fig. 3. Viability, proliferation and MTT conversion to forzam of Mad2 knockdown clones. A.) MEF cells were treated for 16 hours with 0, 200 and 400 ng/ml nocodazole. Shown are percentages of viable wildtype (filled columns) and knockout (empty columns) MEFs stably transduced with shctr, shMad2 #39 or shMad2 #41. n=1. **B.)** Shown are percentages of living cells compared to untreated cells transduced with the respective sh construct. Cell numbers of untreated cells were set to 100%. **(C)** Conversion of MTT to forzam in cells exposed to the indicated concentrations of nocodazole for 16 hours normalized to the values for 1 ng/ml nocodazole.

To investigate the effect of Mad2 depletion upon microtubule depolymerisation, we determined cell growth in nocodazole treated Mad2 knockdown and *Paskin* knockout MEFs. MEF cells were exposed for 16 hours to nocodazole (0 ng/ml, 200 ng/ml and 400 ng/ml). Cell viability was determined by trypan blue exclusion. After 16 hours of nocodazole treatment, we observed a reduced viability in cells exposed to nocodazole. We could not observe any differences in viability depending on genetic background or the transfected sh construct (Fig. 3A). We next determined the amount of living cells compared to untreated cells of each transduced line after 16 hours. We observed a slightly higher proliferation capacity of the Mad2 knockdown cells during nocodazole treatment (Fig 3B). This might be due to a decrease of cells undergoing a mitotic arrest caused by nocodazole. However this effect seems to be independent on the *Paskin* genetic background. Furthermore, we determined the viability of these cells also by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT, a tetrazole is reduced to forzam when incubated with living cells. The amount of forzam produced is proportional to the amount of living cells. Additionally, interleukin 2 or lipopolysaccharide stimulated leukocytes produce more forzam than resting cells (Mosmann 1983). This is due to the fact that the MTT reduction to forzam is depending on NADPH (Berridge and Tan 1993). Before we measured conversion of MTT to forzam, the cells were exposed for 16 hours to 1 ng/ml, 10 ng/ml, 100 ng/ml and 1 µg/ml nocodazole. We found that the effect of increasing nocodazole concentrations on the conversion of MTT to forzam was independent of Paskin (Fig. 3C). Independently of the methods used, we found that proliferation and viability is independent of Paskin. The observed not significant differences in the various clonal pools were probably due to clonal alteration during selection.

Susceptibility of Mad2 depleted cells to DNA damage is independent of Paskin

Mad2 knockdown in MEF cells with a *Paskin* knockout or wildtype background were used to determine susceptibility to DNA damage. In *Drosophila*, depletion of Paskin and Mad2 together is known to increase DNA damage significantly (Shaukat et al. 2012). In mammals, Mad2 increases the susceptibility for DNA damage-induced apoptosis (Cheung et al. 2005; Du et al. 2006; Fung et al. 2006). Additionally, RNAi against Mad2 resulted in reduced mitotic arrest caused by DNA damage (Nitta et al. 2004).

We compared phosphorylation of histone H2AX (γH2AX) in Mad2 depleted MEF cells derived from *Paskin* knockout animals and wild type animals challenged with etoposide. We found no obvious differences in γH2AX staining between *Paskin* knockout and wildtype cells

independently of their Mad2 levels (Fig. 4). This suggests that loss of Paskin does not affect susceptibility to DNA damage even in combination with Mad2 depletion in MEF cells.

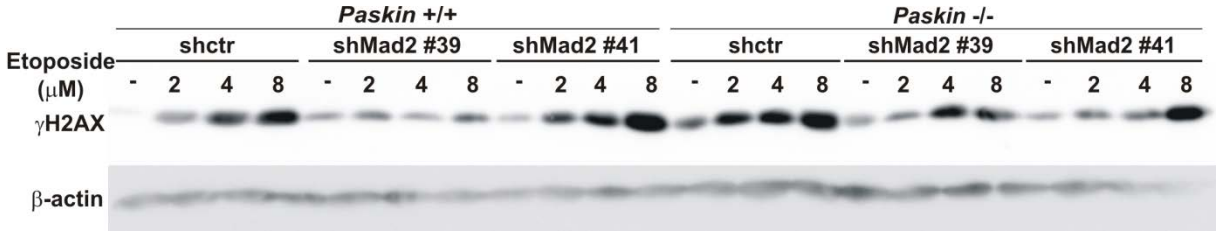

Fig. 4. Etoposide induced accumulation of γH2AX in Mad2 depleted *Paskin* knockout and wildtype MEFs.
Cells were treated for 1 hour with etoposide as indicated. Whole cell lysates were analysed by immunoblotting for γH2AX and β-actin.

Discussion

In *Drosophila*, knockdown of Paskin is known to result in no obvious phenotype. This is very similar to ours and others observation in Paskin knockdown or knockout in mammalian cells and mice, respectively. Only double knockdown of Mad2 and Paskin in *Drosophila* has a lethal effec, whereas in mammals complete loss of Mad2 is sufficient for lethality (Dobles et al. 2000). By restricting the knockdown of Paskin and Mad2 to the wing discs of *Drosophila* Gregory and co-workers were able to investigate the mechanism behind the increased lethality (Shaukat et al. 2012). Double knockdown of Paskin and Mad2 results in an increased DNA damage detected by γH2AX staining and increased p53 dependent apoptosis. We aimed to investigate a putative role for Paskin in the DNA damage response in mammals. We induced double strand breaks in MEFs by treatment with etoposide. Etopside induces double strand breaks by altering the natural cleavage ability of topoisomerase II (Montecucco and Biamonti 2007). The established anti-cancer drug etoposide has been used extensively to investigate the DNA damage response. We could not detect a difference in γH2AX foci formation depending on the *Paskin* genotype. However, we experienced a high basal number of γH2AX foci that could mask a Paskin dependent effect. This could be caused by unspecific binding of the anti-γH2AX antibody or by clonal artefacts due to the isolation of the primary mouse embryonic fibroblasts. However, our results would resemble what was observed in *Drosophila*, where no increase in γH2AX staining could be determined in wing discs depleted of Paskin by RNAi

(Shaukat et al. 2012). In contrast to Paskin, knockdown of Mad2 seems to have a much stronger impact on viability of mammalian cells than of *Drosophila* cells. Also yeast cells with Mad2 deletion are viable under normal growth conditions (Li and Murray 1991). The detrimental effect of Mad2 loss in mammalian cells might explain our initial problems to obtain clones with a stable reduction of Mad2. Although we were finally successful the strong selection conditions over an extended period of time needed to obtain stable clones could also have allowed the accumulation of clone-specific characteristics compensating for reduced Mad2 expression levels. Initial effects of Mad2 reduction might be no longer detectable after such an adaptation. This has to be considered in the interpretation of results gained from experiments with these cells.

Intriguingly, we discovered almost doubled Mad2 protein levels in MEF cells derived from *Paskin* knockout mice compared to wildtype. This effect turned out to be limited to the protein levels, since no changes in mMad2 RNA was observed in these cells. Since the data from *Drosophila* suggest that Mad2 and Paskin cooperate in their function, *Paskin* knockout MEFs might compensate for the loss of Paskin with increased Mad2 levels. To determine the relevance of these observations, Mad2 protein expression has to be analysed in transient Paskin knockdowns in other cell lines. Furthermore, the effect of Paskin overexpression on Mad2 would be of interest. We could observe that the Mad2 depleted cells exhibit less inhibition of proliferation under nocodazole treatment than cells infected with sh control. This makes sense considering that Mad2 depleted mammalian cells challenged with nocodazole have been observed to fail to undergo mitotic arrest (Michel et al. 2001). Although this further confirms that Mad2 knockdown was successful, this effect on proliferation seems to be independent of Paskin. We could observe no differences in the effect of nocodazole on viability depending on Paskin or Mad2 expression. Unexpectedly, we could not observe a difference in γH2AX levels in Mad2 knockdown MEF cells derived from wildtype or *Paskin* knockout mice. Double knockdown of Paskin and Mad2 in *Drosophila* wing discs resulted in a strong increase of γH2AX staining. The differences between our observations and the ones in *Drosophila* might be explained by compensation for the Mad2 reduction. However, it cannot be excluded that a depletion of Paskin and Mad2 in the mammalian system has different effects than in *Drosophila*. Interestingly, it has been reported that Mad2 overexpression negatively influences γH2AX accumulation in response to cisplatin. Cisplatin induces inter- and intra-DNA strand crosslinks and therefore interferes with mitosis (Wagner and Karnitz 2009). This might explain why the knockdown by shMad2 construct #41, that

was more efficient than #39, shows higher γH2AX levels. This effect is slightly reduced in the *Paskin* knockout background. But the relevance of this remains unclear. However, the stable Mad2 knockdown in *Paskin* knockout MEFs might not be the right model to investigate this. These cells might have adapted to the loss of Paskin and Mad2 by regulation of other factors involved in the DNA damage response and therefore do not show the phenotype expected taking in consideration the data from *Drosophila* (Shaukat et al. 2012). Gregory and co-workers do not provide a mechanism for the synergistic effect of Paskin and Mad2 knockdown in *Drosophila* and we could not confirm or exclude a similar mechanism in mammalian cells. Clearer results might be obtained in the mammalian system, which is more sensitive to Mad2 deficiency, by transient knockdown of Mad2. This could avoid accumulation of clonal artefacts. Paskin after all might be involved in the DNA damage response that is tightly regulated by serine/threonine kinases (Lopes et al. 2001; Chen and Sanchez 2004). The observations in *Drosophila* might be explained by a shortened DNA damage induced cell cycle arrest, where Mad2 has been shown to be involved (Dotiwala et al. 2010). The additional knockdown of Paskin might weaken the machinery to an extent sufficient for cells to proceed with the cell cycle before all DNA damage is repaired.

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7 MANUSCRIPT IV (UNPUBLISHED)

The actin cytoskeleton links mammalian PASKIN to cell proliferation

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Abstract

The role of mammalian PASKIN is not well understood, although it has been associated with regulation of cellular energy metabolism and translation. We found that PASKIN mRNA levels are drastically reduced in HL-60 cells under long term culture conditions suggesting that a component the medium is influencing PASKIN mRNA levels. We found that increased FCS concentrations have a negative effect on the regulation of PASKIN mRNA levels indicating that increased proliferation led to a faster depletion of a positive regulatory factor in the medium. Interestingly, we found that amino acid supplementation could delay the decrease of PASKIN mRNA levels. However, the regulation of PASKIN mRNA was depending on the ability of HL-60 cells to proliferate suggesting that proliferation ability rather than an external factor is responsible for PASKIN mRNA level regulation. By immunofluorescence we could show that PASKIN is colocalising with the actin cytoskeleton implicating a putative role for PASKIN associated with proliferation

Introduction

The PAS domain containing serine/threonine kinase PASKIN is the mammalian ortholog of the yeast proteins Psk1 and Psk2, which have been shown to be involved in changes of carbohydrate utilisation upon external stimuli like changes of carbon source or cell integrity stress (Grose et al. 2007). However, the molecular mechanism behind the activation of Psk1

and Psk2 remains unknown. The structural investigations of the PAS domain of the mammalian PASKIN suggest the binding of a ligand that releases and thereby activates the kinase domain, normally inhibited by the PAS domain not binding this ligand (Amezcua et al. 2002). However, the ligand remains unknown till today. It has been suggested that PASKIN expression and activity is regulated by glucose concentration and that mammalian PASKIN is involved in the regulation of insulin as well as glucagon expression and secretion in the pancreas (da Silva Xavier et al. 2004; da Silva Xavier et al. 2011). The regulation of Paskin mRNA levels by glucose could not be reproduced and experiments with pancreatic islets of *Paskin* knockout mice did not confirm that Paskin is necessary for proper insulin expression and secretion (Borter et al. 2007). This makes it more difficult to explain why *Paskin* knockout mice have an altered metabolism leading to changes of their response to increased fat content in their diet (Hao et al. 2007). This is the only phenotype for *Paskin* knockout mice described so far. This phenotype is subtle but we could confirm that *Paskin* knockout mice fed with a high fat diet gain less body weight compared to their wildtype litter mates (Schläfli et al. 2009). However, it is not known which mechanisms are leading to this phenotype. A variety of observations in yeast and mammals suggest a connection between PASKIN and the translational machinery (Rutter et al. 2002; Eckhardt et al. 2007; Schläfli et al. 2011). An involvement of PASKIN in nutrition-dependent regulation of glucagon and insulin translation could explain some of the reported observations. We were interested in the influence of nutrition and growth factor availability *in vitro* on PASKIN mRNA levels. Additionally, we used immunofluorescence to analyse how the intracellular localisation might contribute to PASKIN function.

Materials and Methods

Cell culture and differentiation of cell lines

HL-60 and THP-1 were cultured at 37°C in RPMI (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 50 IU/ml penicillin and 50 µg/ml streptomycin (Sigma). HeLa cells were cultured in DMEM with the same supplements at 37°C. AB81cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FCS (Invitrogen), IU/ml penicillin, and 50 µg/ml streptomycin (Sigma) and insulin-transferrin-selenite supplement (Roche Applied Sciences) at 33°C as described previously (Saleem et al. 2002). Differentiation of AB81 was performed by culturing cells at 37°C for 60 hours. Cells were

split every 48 hours. For long term culture experiments HL-60 cells were seeded and let to attach over night and then grown under normal conditions without splitting the cells for the entire duration of the experiment. Non-essential amino acids (Sigma) were added to final concentration of 2x of the manufacturer's recommendation in the rescue experiments. For differentiation experiments HL-60 cells were diluted with fresh medium to a concentration of 5 x 10^5 cells/ml and were allowed to attach for 16 hours. All-trans retinoic acid (Sigma) was dissolved in ethanol and added to the HL-60 to a final concentration of 1 µM. Control cells were treated with the corresponding amount of ethanol. DMSO (Sigma) was added to a final concentration of 1.25% v/v. Medium containing the described additives was changed after 48 hours for the longer time points. Cells were cultured for the indicated time and collected in solution D (4 M guanidine thiocyanate, 25 mM Sodium citrate pH 7.0, 0.5% Sarcosyl, 0.1 M β-mercaptoethanol). Confirmation of differentiation was done by Wright-Giemsa staining. 200 µl containing 3 x 10^5 suspended cells were added to the cytospin applicator. Cells were centrifuged for 2 minutes at 300 g on cover slides. The slides were dried and treated with 100% methanol for 2 minutes followed by 4 minutes in Wright stain (Sigma). Slides were then submerged in Wright stain diluted 1:5 in Sorensen buffer pH 6.5 (265 ml 0.15M Na_2HPO_4, 735 ml 0.15 KH_2PO_2) for 4 minutes. Slides were stained for 4 minutes in Giemsa stain (Sigma) diluted 1:9 in H_2O. Slides were washed extensively with distilled water and air dried. Cell morphology was analysed by light microscopy.

Immunofluorescence

Glass cover slips in 12 well plates were coated with poly-l-lysine (Sigma) for 30 minutes at room temperature. Poly-l-lysine was removed and slips were dried for 4 hours at room temperature. 5 x 10^5 cells were seeded on coated cover slips. Cells were allowed to attach for at least 16 hours. Cover slips were washed twice with PBS and cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature followed by three washing steps with PBS. To quench free aldehyde groups, cells were incubated for 5 minutes in 20 mM glycine in PBS. Permeabilisation of cells was performed with 0.1% saponine and 20 mM glycine in PBS for 20 minutes at room temperature. Cells were washed three times and blocked with 10% FCS in PBS for 30 minutes at room temperature. The cover slips were incubated with primary antibody against PASKIN (Thermo Scientific) in 0.1% saponine in PBS for 1 hour at room temperature in a humidified atmosphere. After washing 3 times with PBS, cover slips were incubated with secondary antibody against mouse IgG labelled with Alexa 488 in 1% saponine-PBS for 30 minutes at room temperature under humid conditions.

Cells were washed three times with PBS and incubated with DAPI (Sigma), Phalloidin and 0.1% saponine in PBS for 30 minutes at room temperature. After washing with PBS cover slips were mounted with Mowiol (Calbiochem) and dried over night.

RNA extraction and qPCR

Total RNA was isolated as described elsewhere (Chomczynski and Sacchi 1987). 1 µg RNA was reverse transcribed with AffinityScript multiple temperature reverse transcriptase (Agilent) according to the manufacturer's recommendations. cDNA was diluted 1:4 with H_2O. 2 µl cDNA corresponding to 25 ng RNA was used for each reaction. Additionally, each reaction contained 12.5 µl SybrGreen Jumpstart (Sigma), 0.1 µl 100 µM fwd primer and of rev primer, 0.1 µl reference dye and 10.2 µl water. Quantitative PCR was performed with an Mx3000p real-time PCR System (Stratagene). Initial copy numbers were determined by comparison with a standard curve of a serially diluted calibrated standard. To confirm equal loading, mRNA levels were compared to mRNA levels of the internal reference ribosomal protein L28. Analysis of melting curves was performed for all experiments to confirm specific amplification.

Results

PASKIN mRNA levels are reduced in long term cultures of HL-60 cells

We have shown elsewhere that leukocyte cell lines like the human promyelocytic cell line HL-60 show high levels of PASKIN mRNA (manuscript in preparation, chapter 5). Interestingly, we observed in a series of experiments that PASKIN mRNA levels dropped after prolonged culture time without changes of medium. We reproduced these conditions in a controlled manner and found that PASKIN mRNA decreased after 48 to 72 hours under normal cell culture conditions without change of medium or splitting (Fig. 1A) This effect could be reversed when medium was exchanged with fresh medium every 24 hours (Fig. 1B). We found that detaching the cells and reseeding only half of them had only minor effects on PASKIN expression compared to changing the medium (Fig. 1B). This strongly suggests that PASKIN expression is regulated by the increasing or decreasing concentration of an unknown substance in the medium over time.

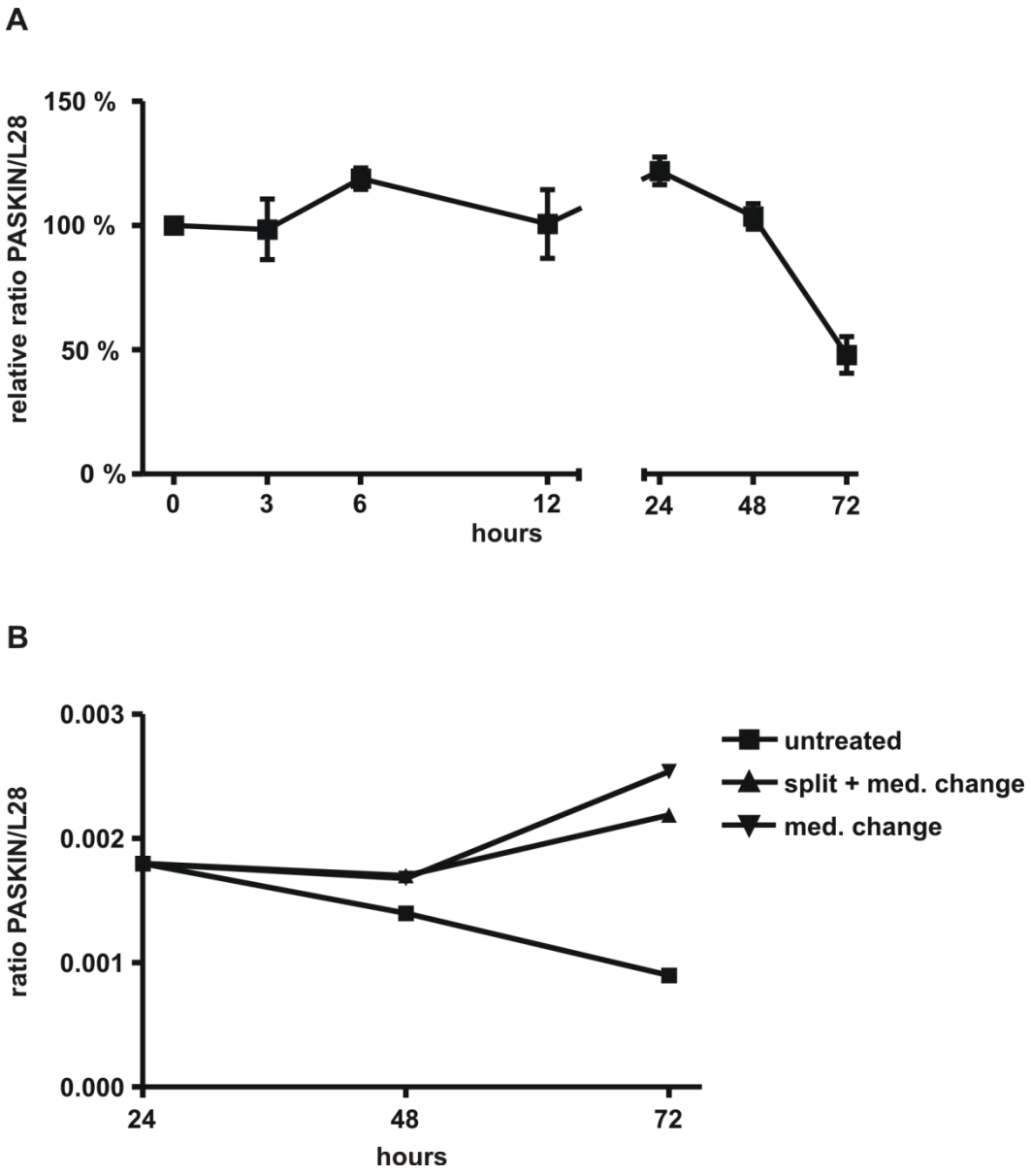

Fig. 1. PASKIN mRNA levels are down-regulated in HL-60 cells in long term culture. A.) The ratios of PASKIN mRNA to L28 mRNA at each timepoint of three experiments were normalized to the coresponding ratio at 0 hours. **B.)** Cells were split and re-seeded with fresh medium or medium was exchanged without splitting every 24 hours after seeding. During every manipulation samples were collected and analysed for PASKIN and L28 mRNA levels. Ratios of PASKIN compared to L28 mRNA levels are shown.

Lower FCS concentrations have a stabilizing effect on PASKIN mRNA levels

It is reasonable to assume that certain growth factors present in the fetal calf serum (FCS), used as medium supplement, influence PASKIN expression in HL-60 cells. To address the question if reduced PASKIN mRNA levels are caused by a loss of one or several growth

factors over time, we cultivated HL-60 cells in medium with different FCS concentrations (0%, 2%, 10% and 20%). Viability of the cultured cells was only affected by the complete absence of FCS resulting in only 78% viability after 72 hours. However, cells cultured in low concentrations (2 and 5%) showed substantially slower growth. This resulted in approximately 10 times higher numbers of cells in the culture with 20% FCS compared to the culture with 0% FCS (Fig. 2A). We measured PASKIN mRNA levels after 24 hours, 48 hours and 72 hours in culture for all the FCS concentration. Interestingly, PASKIN mRNA levels remained relatively stable in cells grown under low FCS conditions.

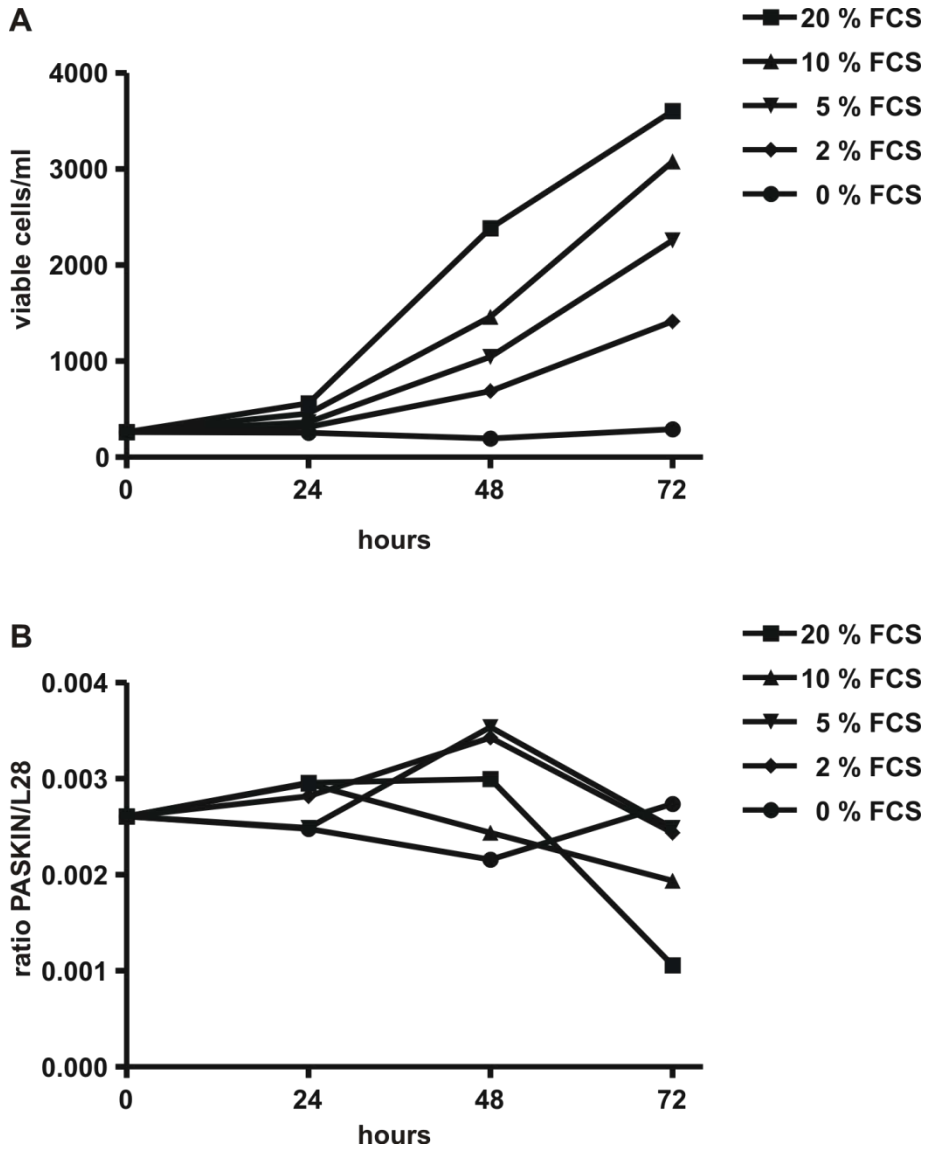

Fig. 2. PASKIN mRNA levels are indirectly dependent on FCS levels. A.) Shown are the viable cells/ml at the time of cell harvesting for the cultures with the indicated FCS concentration. . **B.)** Ratios of PASKIN mRNA to L28 mRNA of the cells shown in **A** for the indicated FCS concentrations are shown.

However, cells grown at 20% and to a lesser extend at 10% FCS lost the ability to sustain PASKIN expression after 72 hours (Fig. 2B). This observation is probably based on the reduced proliferation in medium with low concentrations of FCS. The resulting lower concentration of cells might lead to a slower consumption of a positive regulator of PASKIN mRNA levels or to a faster accumulation of a inhibitory substance involved in negative PASKIN mRNA regulation. The apparent difference to the mRNA levels in cells grown for 72 hours in figure 1 are based on the additional medium change after the 16 hours attaching phase in the experiment shown in figure 2.

Amino acid supplementation delays reduction of PASKIN mRNA levels

Since the yeast orthologs and the mammalian PASKIN have been associated with translational regulation (Rutter et al. 2002; Eckhardt et al. 2007), we were interested in the effect of amino acid availability on PASKIN mRNA levels. To investigate if the loss of amino acids over time causes a reduction of PASKIN mRNA levels, we added amino acids to long time cultures after 48 hours and 72 hours. Intriguingly, adding amino acids at 48 hours had a similar effect on PASKIN mRNA expression as fresh medium supplementation. However, fresh medium could keep PASKIN mRNA levels high for a longer time period than the amino acid mixture (Fig. 3).

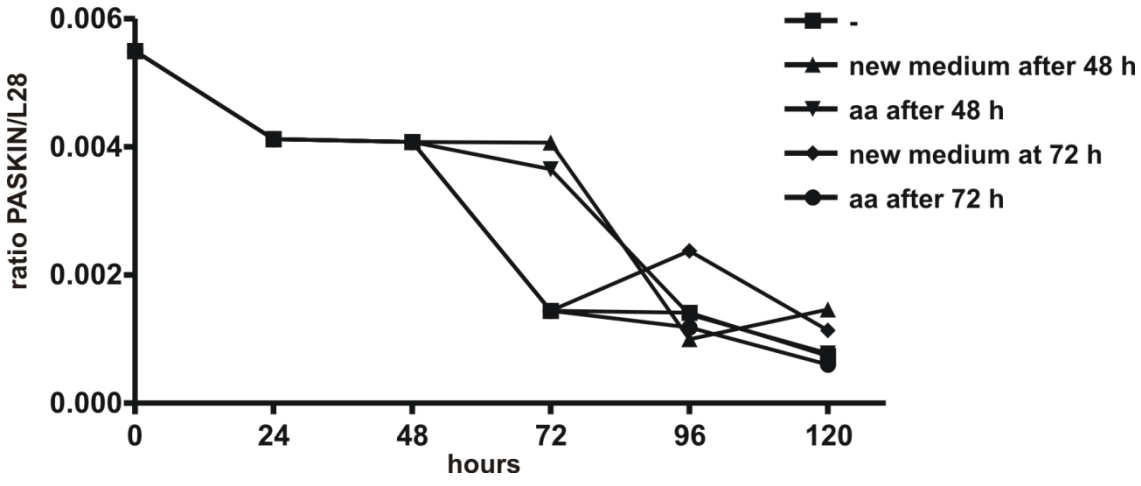

Fig. 3. Amino acid supplementation protects HL-60 from loss of PASKIN mRNA levels
The indicated manipulations were performed at the indicated time. mRNA levels of PASKIN and L28 were determined after the indicated time by qPCR. Results are shown as ratios of PASKIN compared to L28 mRNA levels.

These observations suggest that amino acids are partially able to influence PASKIN mRNA levels. Since FCS can be utilised as a source for amino acids we assume that the additional proliferation induced by FCS let the cells reach faster critical concentrations were additional amino acids from the FCS do not influence PASKIN mRNA levels anymore. The fact that neither amino acids nor fresh medium could keep PASKIN mRNA high for more than 96 hours supports this explanation. This would suggest that PASKIN mRNA levels are influenced by the proliferation ability of the cells.

PASKIN expression is reduced in differentiated HL-60 cells

The negative influence of long term culture on PASKIN mRNA levels could be due to loss of proliferation rate. To investigate the effect of impaired proliferation on PASKIN mRNA expression we exposed HL-60 cells to DMSO and all-trans retinoic acid (ATRA).

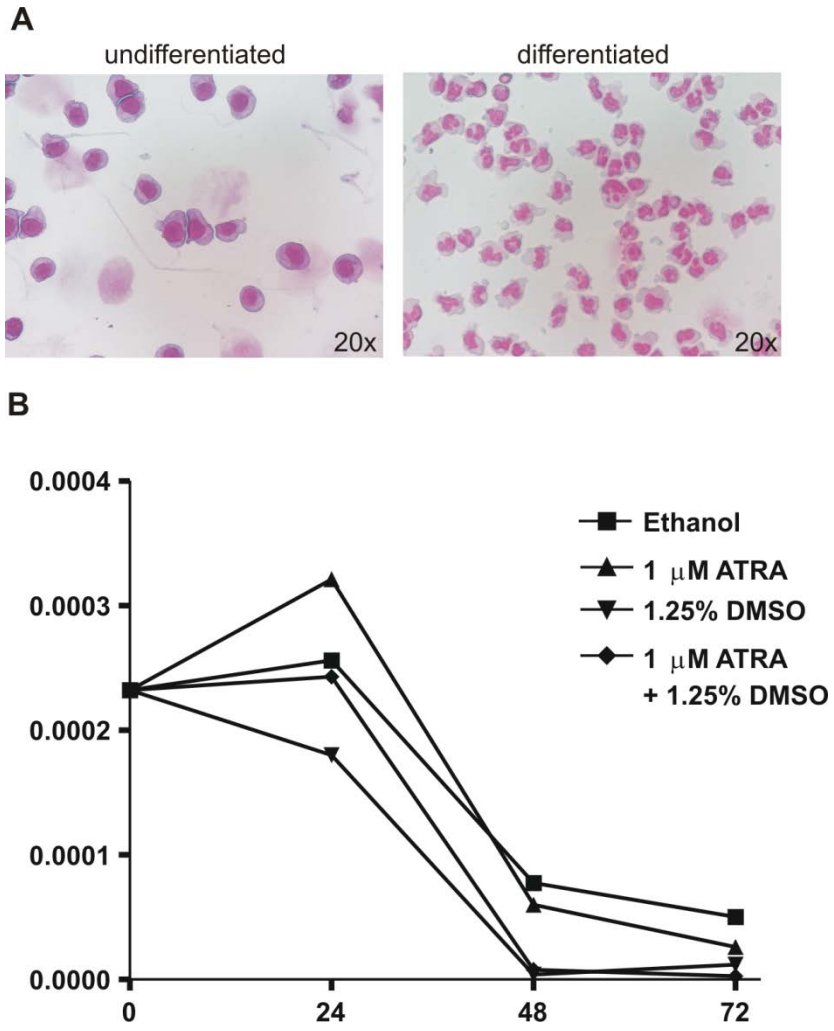

Fig. 4. PASKIN mRNA levels are dependent on the differentiation status of HL-60 cells A.) Wright-Giemsa staining of untreated HL-60 cells and HL-60 cells differentiated with 1 µM ATRA and 1.25% DMSO. **B.)** Ratios of PASKIN mRNA compared to mRNA of L28 in untreated HL-60 and HL-60 exposed to 1 µM ATRA and/or 1.25% DMSO.

It has been shown that DMSO and ATRA or a combination of both lead to differentiation of the predominantly premyelocytes in HL-60 culture to a granulocyte kind of cell type and goes in line with a reduced ability to proliferate but only a minor loss of viability (Breitman et al. 1980). Intriguingly, differentiation of HL-60 reduced PASKIN mRNA even more effectively than long term culture (Fig. 4). This indicates that PASKIN expression goes in line with proliferation ability.

PASKIN colocalises with actin cytoskeleton in different cell lines

The regulation of PASKIN mRNA levels by increased transcription or mRNA stability depending on the proliferation ability of HL-60 cells might indicate that PASKIN is involved in a process involved in proliferation. PASKIN has been previously associated with translation and the mitotic checkpoint (Eckhardt et al. 2007; Shaukat et al. 2012). To learn how proliferation rates affect PASKIN mRNA levels, information about the intracellular localisation of PASKIN might be important. Since HL-60 do not adhere spontaneously we used the monocytic cell line THP-1 instead (Tsuchiya et al. 1980). Although THP-1 is also a suspension cell line it is possible to induce adherence with lipopolysaccharide (LPS) stimulation (Kounalakis and Corbett 2006). Importantly this does not interfere with the viability of THP-1 and LPS does not change the high PASKIN mRNA levels in this cell line (manuscript in preparation, chapter 5). We stained THP-1 cells stimulated with 1 µg/ml LPS for PASKIN. Furthermore, we used labelled phalloidin to stain for filamentous actin. phalloidin is a toxin from *Amonita phalloides* that has been shown to bind to and stabilize F-actin (Dancker et al. 1975). We found PASKIN concentrated at filopodia-like cell extensions and distributed in the cytoplasma (Fig. 5A). Most of the PASKIN signal colocalised with the phalloidin staining for F-actin. This might suggest that PASKIN function is connected to the cytoskeleton. To answer the question if this is a monocyte-specific phenomenon, we stained also HeLa cells for PASKIN and with phalloidin. As expected, the adherent HeLa cell line did not show any filopodia-like structures and phalloidin staining was distributed all-over the cytoplasma (Fig 5B). Confocal microscopy revealed that on the lowest layer phalloidin staining was stronger at focal adhesion points. Interestingly, we also observed that most of the PASKIN signal colocalised with the signal for phalloidin. It might be relevant that some PASKIN signal was not colocalised with the phalloidin staining. This supports association of PASKIN with the cytoskeleton independently of the cell type. To confirm these findings we

used the podocyte cell line AB-81. At 33°C culture conditions AB-81 cells proliferate at a normal rate. However, switching to 37°C AB-81 cells differentiate to podocyte like cells.

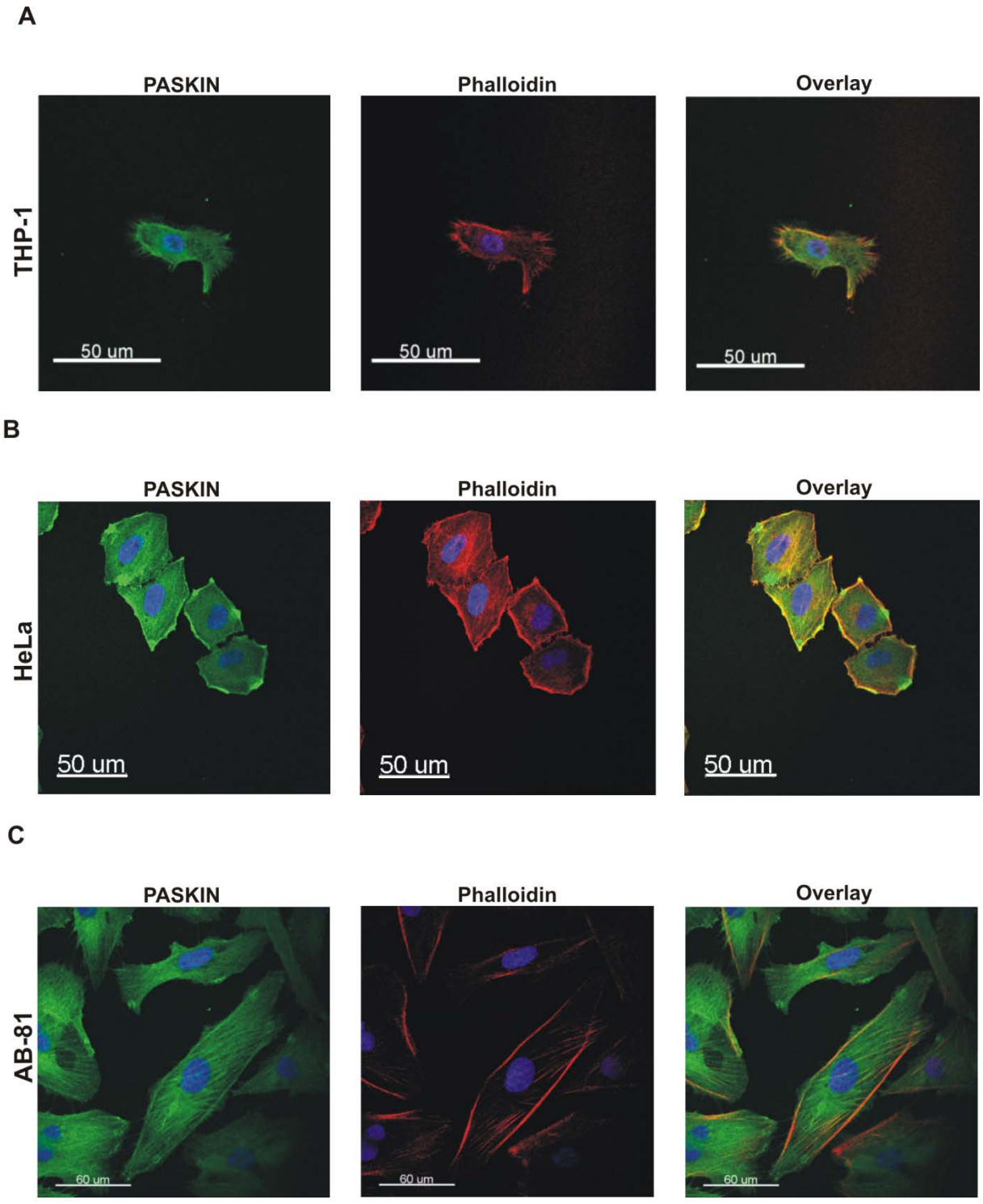

Fig. 5. PASKIN colocalises with actin in various cell lines A.) Adherence of THP-1 cells was induced by LPS stimulation. Shown are the signals of anti-PASKIN and phalloidin staining and the overlay of both signals as indicated. Blue colour indicates DAPI staining. **B.)** HeLa cells were stained as described in A. **C.)** Differentiated AB-81 cells were stained as described in A.

The differentiation of AB-81 cells goes in line with cytoskeletal changes resulting in the typical filopodia structures of *in vivo* podocytes (Saleem et al. 2002). Staining for PASKIN showed clear colocalisation along the cytoskeleton in differentiated AB-81 cells but also a diffuse cytoplasmatic signal could be detected similar to the other cell lines (Fig. 5C). This confirmed a connection of PASKIN with F-actin.

Discussion

The present study demonstrates that PASKIN mRNA levels are reduced in HL-60 cells in long term cultures (more than 72 hours in the same medium) suggesting the PASKIN mRNA levels are regulated by changes of the medium composition. We found that the FCS concentration in medium rather accelerates the reduction PASKIN mRNA levels in HL-60 and that charcoal stripped FCS has no effect on PASKIN mRNA levels (data not shown) suggesting that not the disappearance of a growth factor but rather the increased cell number after 72 hours is responsible for the reduction of PASKIN mRNA levels. This might be by the production of a inhibiting or the consumption of a stimulating factor influencing PASKIN mRNA levels. It was previously shown that glucose concentration can increase PASKIN mRNA and protein expression in the mouse pancreatic cell line MIN6 and in pancreatic β-cells of rat pancreatic islets (da Silva Xavier et al. 2004). Reduced glucose concentration in the cell medium in long term cultures would explain why PASKIN mRNA expression decreases. We could not confirm this observation in mouse pancreatic β-cells (Borter et al. 2007). In this context it is intriguing that glucose concentration has been shown to stimulate proliferation of β-cells of isolated rat islets (Swenne 1982). Furthermore, glucose infusions lead to increased β-cell mass in mice (Bonner-Weir et al. 1989). Induction of PASKIN expression by stimulation of proliferation rather than by glucose directly, would explain the contradictory observations on PASKIN expression in β-cells. PASKIN might then act as a sensor for optimal conditions for proliferation which are already present in cell cultures explaining why increased FCS did not increase PASKIN mRNA. We could show for the first time that amino acids have a positive effect on PASKIN mRNA levels. This is especially of interest considering the links of PASKIN to the translation machinery. We showed previously that PASKIN phosphorylates the translational elongation factor eEF1A1 and that PASKIN increases translation efficiency *in vitro* (Eckhardt et al. 2007). Intriguingly, several phosphorylation targets of the two yeast homologs of PASKIN are associated with

translational regulation (Rutter et al. 2002). Additionally, we could recently show that ribosomal protein S6 is phosphorylated by PASKIN (Schläfli et al. 2011). Although this suggests a connection between PASKIN and proliferation by translational regulation, PASKIN might also be involved in the cytoskeleton organisation. Here, we could demonstrate that PASKIN mostly colocalises with F-actin. Intriguingly, a recent study in *Drosophila* implicated Paskin in the processes around the spindle assembly checkpoint (SAC). In a screen of the *Drosophila* kinome it was found that PASKIN knockdown in combination with a weakening of the SAC leads to increased DNA damage and p53 dependent apoptosis (Shaukat et al. 2012). Assuming a putative influence of PASKIN on actin organisation this is not surprising, since a proper function of the actin cell cortex controls spindle positioning (Cowan and Hyman 2007; Schuh and Ellenberg 2008). An involvement of PASKIN in actin organisation during cell division would explain why PASKIN mRNA levels are reduced in HL-60 losing their ability to proliferate.

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8 CONCLUSIONS

The yeast orthologs of mammalian PASKIN, Psk1 and Psk2, are activated upon cell integrity stress by compounds such as sodium dodecyl sulphate (SDS), calcofluor white or chlorpromazine (Grose et al. 2007). Additionally, Psk1 and Psk2 have been associated with the adaptation to changes in the carbon source of the growth medium (Rutter et al. 2002; Grose et al. 2007). Cell integrity stress and growth on a non fermentative carbon source lead to increased kinase activity of Psk1 and Psk2 and subsequently to increased phosphorylation of UDP-glucose-pyrophosphorylase (Ugp1). This phosphorylation has been proposed to be important for redistribution of glucose from the storage carbohydrate glycogen to the structural carbohydrate glucan which is an important part of the yeast cell wall. This switch is achieved by directing Ugp1 to the cell periphery rather than changing Ugp1 activity. Additionally, the phosphorylation of Ugp1 at serine 11 also triggers the formation of a signalling complex that is able to activate Rho1 (Cardon et al. 2012).

In contrast to yeast, the function and regulation of mammalian PASKIN is still very poorly understood. The putative role of PASKIN in the α- and β-cells in the islets of Langerhans is highly disputed due to contradictory data (da Silva Xavier et al. 2004; Borter et al. 2007). The effect of glucose on the PASKIN mRNA is varying from induction to inhibition in different tissues. Moreover, in different laboratories the effects of glucose on the same cell lines and cell types have been reported to be variable. This can be explained by variation in laboratory techniques, but it could be also a hint that glucose is not the most important factor influencing PASKIN regulation. It is more likely that glucose in certain tissue and under certain conditions induces a signalling cascade upstream of PASKIN but the same signalling cascade is activated by other signals under other circumstances. The low mRNA levels in the pancreas might be due to a masking effect of cells that do not express PASKIN. β-galactosidase staining in isolated islets from *Paskin* knockout mice expressing a *Paskin*-lacZ fusion mRNA under the endogenous *Paskin* promoter, did not indicate that Paskin is expressed in a relevant amount (Borter et al. 2007).

Similar controversial data have been reported on the downstream effects of Paskin (da Silva Xavier et al. 2004; Borter et al. 2007). Loss of Paskin seems to partially protect mice from the metabolic syndrome under high fat feeding conditions (Hao et al. 2007; Schläfli et al. 2009). Additionally, a *PASKIN* mutation isolated from a family with increased frequency of early onset diabetes showed an increased kinase activity that leads to a higher basal insulin

secretion (Semplici et al. 2011). On the other hand, overexpression of PASKIN has been shown to protect cells from the detrimental effects of glucolipotoxicity (Fontés et al. 2009). It is important to note that for all of these observations no direct and reproducible mechanism has been provided. Considering mRNA and protein levels in various mice tissues supports the idea that the role of Paskin has to be found in other tissues than the pancreas. The high expression of Paskin in testis might suggest a role for Paskin in sperm maturation. However, no impairment of sperm development or motility has been observed (Katschinski et al. 2003; Eckhardt et al. 2007). In line with this observation, *Paskin* knockout mice show normal litter sizes. The high mRNA levels of Paskin in mouse tissues of the immune system have been observed early on but no functional connection has been made. We assumed that the high levels of Paskin in these tissues are of functional importance and investigated a possible reason in more details.

Putative functional indications by the high PASKIN mRNA in leukocytes

We found that the tissue-specific regulation of *Paskin* gene expression is evolutionary conserved between mouse and human. This evolutionary conservation of high mRNA levels in tissues associated with the immune system further supports our hypothesis of a functional role for PASKIN in this compartment. We assumed that the high PASKIN levels in the lymph nodes and the bone marrow are due to high denovo transcription of PASKIN in immune cells. Indeed, we found PASKIN is highly expressed in peripheral mononuclear cells (PBMCs). Especially, B- and T-cells show high PASKIN mRNA levels. This is interesting because of the functional and developmental similarities between these two cell types, as they both derive from the same precursor cell. B-and T-cells express antigen specific receptors. This might indicate that PASKIN is involved in one of the many shared processes in this cell lineage. We looked at the effect on PASKIN mRNA levels of stimulation of T- and B-cell lines of mouse and human origin. Interestingly, we found that PASKIN mRNA levels are very stable in response to this kind of stimulation in leukocyte cell lines. To exclude any cell line artefact we also investigated the effect of immunostimulants and inhibitors in primary PBMCs with similar results. This might suggest that it is biologically important to have high steady state levels of PASKIN mRNA in lymphocytes. In this regard, PASKIN regulation could be similar to the regulation of T-cell proteins CD3, CD4 and CD8 that are known not to be altered upon T-cell activation (Teague et al. 1999). However, to exclude that RT-qPCR on

RNA isolated from mixed and isolated cultures of primary cells is not an adequate method to detect a regulation of PASKIN, we designed FACScanning experiments to overcome putative weaknesses of the previous experimental design. The labelling of the different cellular subsets together with PASKIN allowed us to observe changes of protein levels in the different subsets in the context of other cell types. We found that PASKIN is not affected by the pro-inflammatory stimuli in any of the cell subsets. We did not investigate in more detail how the activation process is affected by the loss of PASKIN. The experiments done in the laboratory of Katapodis at Novartis Basel, show weak differences in *Paskin* knockout mice compared to wildtype. These results suggest an involvement of Paskin in B-cell proliferation and T-cell differentiation although the variations between different mice make this difficult to interprete. The minimal differences in the *Paskin* knockout mice might be due to an adaptation to a chronic systemic loss of Paskin. Therefore it could be of interest to investigate how a temporary knockdown of PASKIN affects lymphocyte activation. It is important to keep in mind that both B- and T-cells react with increased proliferation upon stimulation. Proliferation is a returning topic in the discussion about the function of PASKIN. Indeed PASKIN mRNA levels seem to correlate with proliferation ability also in the premyeloid cell line HL-60.

Loss of proliferation ability leads to reduced PASKIN mRNA levels

In addition to the T-cell line Jurkat and the monocytic leukemia cell line THP-1, also the premyeloid cell line HL-60 expresses high levels of PASKIN mRNA. Interestingly, HL-60 cells in long term cultures start to reduce the levels of PASKIN mRNA. We first observed this when we stimulated HL-60 for several days with LPS. We were able to delay the decrease of PASKIN mRNA by adding amino acids to the culture medium at later time points, whereas the concentration of fetal calf serum (FCS) did not have an effect on PASKIN mRNA levels. Currently, we do not have an explanation for this observation. However, we found that the differentiation of HL-60 to granulocytes had a similar effect on PASKIN mRNA levels. The differentiation of HL-60 goes in line with a reduced ability to proliferate (Breitman et al. 1980). Taken together, these data indicate that PASKIN mRNA levels are high when there is a high potential for a fast increase of proliferation and low when the cells lose their ability to proliferate. However, this is speculation and it is unclear if this observation is of any relevance. It could be that PASKIN is phosphorylating and therefore regulating proteins

involved in proliferation or regulation of cell cycle progression. Interestingly, a study in *Drosophila* brought Paskin in the context of Mad2 and the spindle assembly checkpoint.

Is there a link between PASKIN function and the spindle checkpoint?

The data obtained in *Drosophila* suggest that reduced Paskin levels might increase susceptibility to DNA damage in a Mad2 depleted background. The group of Stephen Gregory at the University of Adelaide, Australia, observed that double knockdown of Mad2 and Paskin resulted in increased p53-dependent apoptosis due to increased DNA damage (Shaukat et al. 2012). Although Mad2 knockdown results in a sloppy chromosome distribution, observable by lagging chromosomes it seems not to induce the DNA damage response. Similarly, Paskin knockdown alone does not result in an increase of phosphorylation of histone H2AX (γH2AX), which is an early step of the DNA damage response. However, when Paskin is depleted together with Mad2 more cells undergo apoptosis (Shaukat et al. 2012). Since this effect is only apparent when both Paskin and Mad2 are depleted it is unlikely that they directly interact. However, they might have a role in parallel processes, and interfering with both of them simultaneously results in increased DNA damage and subsequent apoptosis. In our experiments, we induced DNA damage in *Paskin* knockout mouse embryonic fibroblasts (MEFs) and wildtype MEFs with etoposide. Staining for γH2AX suggests that etoposide induced DNA damage is independent of Paskin. Additionally, immunoblots for phosphorylated histone H2AX in *Paskin* knockout MEFs depleted of Mad2 suggest no effect of *Paskin* genotype on the DNA damage response. Therefore, our results do not confirm the observations made in *Drosophila*. However, in *Drosophila*, the DNA damage was not induced but rather occurred spontaneously. We observed that γHA2X is independent of Paskin and Mad2 in untreated MEFs. This might be due to a certain redundancy in mammalian cells compensating for the loss of Paskin or Mad2 and therefore provide more protection of DNA damage compared to *Drosophila* cells. Furthermore, the stable knockdown of Mad2 and the long term selection involved might have resulted in an adaptation to the Mad2 depletion reducing an observable effect. Therefore, the observations in *Drosophila* should be considered as an important hint for Paskin function. It remains however unclear what is the mechanism behind the increased DNA damage in Paskin/Mad2 double knockdowns.

In eukaryotes, DNA double strand breaks triggers cell-cycle arrest. This is achieved via the DNA-damage checkpoint. The first step of the signalling cascade resulting in mitotic arrest is the recognition of single-stranded DNA (ssDNA). A complex built by ataxia-telangiectasia mutated- and Rad3-related (ATR) and ATR-interacting protein (ATRIP) recognises replication protein A (RPA) binding to ssDNA (Zou and Elledge 2003). ATR is a serine/threonine kinase able to phosphorylated histone H2AX in response to DNA damage (Ward and Chen 2001). The phosphorylation of H2AX is involved in the recruitment of the DNA damage repair machinery (Downs et al. 2000). An additional function of ATR is to trigger cell-cycle arrest. This involves the kinases Chk1 and Chk2 (Lopes et al. 2001; Chen and Sanchez 2004). Chk1 phosphorylates securin and thereby inhibits its ubiquitination and subsequent degradation (Wang et al. 2001). The same is achieved the interaction of the anaphase promoting complex with securin (Agarwal et al. 2003). The APC dependent ubiquitination of securin is based on the direct interaction of CDC20 as a part of the APC and securin. Chk2 is probably inhibiting the interaction by phosphorylation of CDC20 (O'Neill et al. 2002). In addition, protein kinase A (PKA) is inhibiting CDC20 by phosphorylation in an ATR dependent way (Searle et al. 2004). CDC20 is also a target of MAD2 during the spindle assembly checkpoint. Interestingly, it has been shown that deletion of MAD2 reduces the time during which cells stay in arrest due to double strand breaks (Dotiwala et al. 2010). Under normal conditions DNA damage-induced cell cycle arrest is resolved when double strand breaks have been repaired or the cell adapts to the DNA damage (Yoo et al. 2004; Deckbar et al. 2007). Reduced time to repair the DNA before adaptation will result in an accumulation of DNA damage. PASKIN might be involved in maintaining cell cycle arrest caused by DNA damage and therefore a knockdown of PASKIN might decrease adaptation time and lead to increased accumulation of DNA damage.

The bidirectional character of the PASKIN-protein phosphatase 1 regulatory subunit 7 promoter

The genomic localisation of the human *PASKIN* and the protein phosphatase 1 regulatory subunit 7 (*PPP1R7*) suggests that they share a common promoter region. The two genes are only approximately 1 kilobase (kb) apart and they are arranged head to head. We found that this arrangement is conserved in all tetrapodes and in the Sarcopterygii *Latimeria chalumae*. Actinopterygii did not show this arrangement nor do lower Animalia. It has been shown

before that the frequency of occurrence of bidirectional promoters like the one of *PASKIN* and *PPP1R7* increases with the complexity of organisms (Koyanagi et al. 2005). It was suggested that in humans about 20% of the genes are organised within 1 kb from each other like *PASKIN* and *PPP1R7* (Adachi and Lieber 2002). Li et al. reported that bidirectional gene pairs are more likely to perform similar molecular functions or are associated with the same biological process or cellular compartment than random gene pairs (Li et al. 2006). For example the glutamine 5′phosphoribosylpyrophosphate amidotransferase (GPAT) and 5′-phosphoribosylaminoimidazole carboxylase (AIRC) are controlled by a bidirectional promoter and catalyse two different steps in purine synthesis (Gavalas and Zalkin 1995). The kinase activity of PASKIN and the regulatory function on a phosphatase of PPP1R7 provide a molecular functional connection. The coexpression of PASKIN and PPP1R7 we found in a series of cell lines further supports the idea of a shared biological progress. The protein phosphatase 1 ortholog in yeast, Glc1 has been shown to be involved in glycogen synthesis and cell wall integrity. These are the same processes the yeast PASKIN orthologs Psk1 and Psk2 are associated with. However, the yeast ortholog of PPP1R7, Sds22, although interacting with Glc7 in other processes, involvement of Sds22 has not been investigated in these aspects of Glc7 function to our knowledge. Sds22 has benn shown to be important for the nuclear functions of Glc7 in chromosome organisation (Ohkura and Yanagida 1991; Peggie et al. 2002). It is interesting that bidirectional promoters of a length of 500 bp or less have been associated with genes involved in DNA repair (Adachi and Lieber 2002; Trinklein et al. 2004; Wakano et al. 2012). Longer bidirectional promoters are associated with chromosome organisation and stability (Li et al. 2006; Wakano et al. 2012). This is remarkable in view of the fact that in *Drosophila* Paskin has been directly linked to increased DNA damage in a chromosome instability background (Shaukat et al. 2012).

Our studies on the *PASKIN-PPP1R7* promoter constructs revealed that the activity of the promoter in *PASKIN* orientation is much lower than in *PPP1R7* orientation under standard culture conditions. Since the mRNA levels of PASKIN and PPP1R7 positively correlate in different cell lines, we do not think this indicates a negative correlation of the expression of the two genes, which would be not an uncommon observation in bidirectional promoters (Trinklein et al. 2004; Li et al. 2006). The lower activity in PASKIN direction of the promoter in the reporter constructs might just reflect the lower Paskin mRNA levels observed before (Katschinski et al. 2003). Assuming that there is a coregulation because of a shared biological process, this might indicate that less PASKIN than PPP1R7 is needed in this process or further differential regulation on the protein translational or stability level. We found that the

activity of the promoter in *PASKIN* orientation in the reporter constructs is increased in HL-60 and Jurkat cells compared to the promoter in *PPP1R7* orientation. This further supports a role for PASKIN in leukocytes as we suggested before. However, without clarifying the functional role of PASKIN it is difficult to explain why leukocytes and especially lymphocytes have a special need for PASKIN. We identified an inhibitory element in the *PASKIN-PPP1R7* promoter. This element seems to be responsible for the reduced activity of the promoter in *PASKIN* orientation. It is interesting to note that the *PASKIN-PPP1R7* inhibitory element is localised at a position of equal distance from the two genes. This could suggest an insulator function or an orientation dependent inhibitory element. We found in our reporter construct experiments that the inhibitory element is not differentially active in the four cell lines we investigated. This suggests that it is not causing the relative differences in the regulation of PASKIN and PPP1R7 expression in leukocytes. However, epigenetic modifications or long distant enhancer effects might influence the inhibitory activity of this element. These effects would not be visible in the luciferase reporter assay. The transcriptional regulation of PASKIN by glucose is not well understood and could not be reproduced by us. Therefore up to date no reliable transcriptional activator is known for PASKIN. However, the bidirectionality of the *PASKIN-PPP1R7* promoter could indicate that the two genes are involved in the same biological process.

Assuming that PASKIN and PPP1R7 are involved in the same biological process

Although we found that the organisation of *PPP1R7* and *PASKIN* in a bidirectional promoter is evolutionary a rather recent event, the functional similarities between PPP1R7 and PASKIN can be observed already in yeast. Interestingly, the yeast protein phosphatase 1 ortholog Glc7 has been reported to be involved in cell cycle arrest resolving. It has been shown that Sds22, the yeast ortholog of PPP1R7, is involved in the nuclear localisation of Glc7 in this process (Peggie et al. 2002). Sds22 seems to perform this task in concert with Ypi1. Depletion of one of these two proteins leads to a similar inhibition of cell growth (Pedelini et al. 2007). Assuming a similar role for Sds22 and Ypi1, it is remarkable that Ypi1 overexpression leads to reduced glycogen accumulation in yeast (García-Gimeno et al. 2003). Regulation of carbohydrate utilisation was one of the first functions proposed for the yeast PASKIN orthologs (Rutter et al. 2002). Depletion of Ypi1 in yeast leads to activation of the

spindle assembly checkpoint and cell cycle delay (Bharucha et al. 2008). Furthermore, the catalytic subunit Glc7, that is in some cases regulated by Sds22, is involved, among others, in the spindle assembly checkpoint, glycogen metabolism and translation (Wek et al. 1992; Ramaswamy et al. 1998; Cairo et al. 2013). PASKIN has been associated with all of these processes. In mammals, the coregulation of PASKIN and PPP1R7 indicates that the two genes are functionally connected. The function of the human PPP1R7 is not well understood. However, our data and data from other groups investigating other organisms provide hints that PPP1R7 is associated with similar processes as are thinkable for PASKIN. PASKIN mRNA levels are high in testis. The same is true for the PPP1R7 (Katschinski et al. 2003). For PASKIN no functional role in testis is known so far. PPP1R7 has been shown to bind and inhibit the testis-specific protein phosphatase 1 gamma 2 isoform (PP1γ2) (Huang et al. 2002). PP1γ2 is involved in controlling sperm motility during sperm morphogenesis (Chakrabarti et al. 2007). However, PPP1R7, as well as PASKIN, is expressed ubiquitously in higher eukaryotes and its role in regulating PP1 in mitosis is conserved. It has been shown that PPP1R7 is important for the targeting of PP1 to the kinetochores (Posch et al. 2010). PPP1R7 together with PP1 regulates the kinetochore microtubule interaction by counteracting Aurora B. (Wurzenberger et al. 2012). Depletion of PPP1R7 results in increased number of cells with a lagging chromosome and a general delay in cell division (Posch et al. 2010). Activity of Aurora B is depending on the tension at kinetochores and ensures that cycle progression is delayed before proper tension is established (Vázquez-Novelle and Petronczki 2010). It is involved in recruiting components of the mitotic-checkpoint to the kinetochore upon tension loss (Famulski and Chan 2007). Interestingly, Aurora B has been shown to affect proper localisation of Mad2 (Lens et al. 2003). A putative involvement of PASKIN in the cell cycle control during the DNA damage response checkpoint or the spindle assembly checkpoint might depend on a nuclear localisation of PASKIN, at least at some point of the cell cycle. Indeed, PASKIN has been identified in a large-scale screen in HeLa cells for nuclear phosphoproteins (Beausoleil et al. 2004). Others reported a diffuse localisation for PASKIN mainly in the cytoplasm, although it has been detected in the nucleus by immunoblotting of fractionated cells (Rutter et al. 2001). Additionally, PASKIN shows a nuclear-like pattern in spermatogonia (Eckhardt et al. 2007). This suggests strongly that PASKIN can localize to the nucleus in some cases.

Interestingly, we found that PASKIN seems to colocalise also with the actin cytoskeleton. In immunofluorescence staining of a series of cell lines we found PASKIN to accumulate at

focal adhesion points and stress fibre like structures. This brings new ideas about the function of PASKIN. Especially, it is of interest to considering another important function of PPP1R7 in *Drosophila*: the regulation of the dephosphorylation of the ezrin, radixin and moesin (ERM) family members. Loss of PPP1R7 in *Drosophila* leads to disruption of cell polarity (Grusche et al. 2009). Activated ERM proteins are able to bind to transmembrane proteins and to F-actin, and therefore linking the cytoskeleton to the plasma membrane. The ERM proteins are activated by phosphorylation at a conserved threonine residue in the C-terminus (Fievet et al. 2004). Additionally they are activated by binding phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). The ERM family members have a conserved C-terminal actin binding domain. Phosphorylation at threonine 567 of ezrin is important for its role in cytoskeletal rearrangements. The conserved phosphorylation site of the entire ERM family resembles the predicted phosphorylation site consensus for PASKIN proposed before (Kikani et al. 2010; Schläfli et al. 2011). Increased ezrin protein levels in spermatozoa has been associated with human infertility (Salvolini et al. 2013).

An additional link to PASKIN might be the involvement of ERM family proteins in the immunological synapse. It has been shown that changes of threonine phosphorylation status of ERM family members at the immunological synapse are involved in receptor clustering induced by T-cell activation (Faure et al. 2004). ERM proteins interact with CD43 and the cytoskeleton and promote CD43 relocalisation upon T-cell activation (Allenspach et al. 2001). This relocalisation is important for proper T-cell activation and depends on the phosphorylation of moesin (Delon et al. 2001). Another obvious connection between ERM family functions and PASKIN is the secretion of insulin. Glucose stimulation of β-cells leads to a phosphorylation dependent relocalisation of ERM to the cell periphery adjacent to the site of insulin granule accumulation (Lopez et al. 2010). Interestingly, the phosphorylation of ERM proteins depends on the intracellular increase of Ca^{2+} induced by glucose. Lopez and coworkers could show that the secretion of insulin depends on ezrin function (Lopez et al. 2010). Although there is no experimental evidence that PASKIN is involved in regulating the function of ERM proteins, the connections are obvious. A model with putative interaction of PASKIN, PPP1R7 and ERM proteins would provide a link between many observations made for PASKIN. Additionally, it would provide an explanation for the coregulation of PASKIN and PPP1R7. Interestingly, ERM proteins were not found in the yeast genome (Bretscher et al. 2002). Maybe this explains why the function of mammalian PASKIN is difficult to bring in line with the function of its yeast orthologs.

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9 CURRICULUM VITAE

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EDUCATION

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10 CONTRIBUTIONS TO THE THESIS

Unpublished manuscripts:

Egger, S.S., Rückert, B., Spielmann, P., Akdis C.A. and Wenger, R.H. “Regulation of PASKIN levels during leukocyte activation”

All figures.

Egger, S.S., Spielmann, P., Hoogewijs, D. and Wenger, R.H. “Evolutionary conservation of PASKIN and its genomic locus and the consequences on PASKIN and PPP1R7 expression”

All figures.

Egger, S.S., Spielmann, P., Gregory S.L. and Wenger, R.H. “Weakening of the spindle checkpoint by Mad2 depletion in mammalian *Paskin* knockout cells”

All figures.

Egger, S.S., Schläfli, P., Spielmann, P. and Wenger, R.H. “The actin cytoskeleton links mammalian PASKIN to cell proliferation”

All figures except Fig. 5C.

Additional publications not included:

Muriel R. Kaufmann, Sandra Barth, Uwe Konietzko, Bei Wu, **Sascha Egger**, Reiner Kunze, Hugo H. Marti, Meike Hick, Ulrike Müller, Gieri Camenisch, and Roland H. Wenger (2013). "Dysregulation of Hypoxia-Inducible Factor by Presenilin/γ-Secretase Loss-of-Function Mutations." *The Journal of Neuroscience* 33(5): 1915-1926.

Isolated total RNA from animal organs.

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